

**The Effects Of Aldosterone On Sodium  
Transport In Cultured Renal (A6) Cells**

**KIERON BALDWIN**

Presented for the Degree of  
Master of Science in the  
Department of Anatomy and Cell Biology  
Faculty of Medicine  
University of Cape Town

July 1996

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

## DECLARATION

I, **Kieron Shane Baldwin**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

**Signed**

12/07/1996

# CONTENTS

## Acknowledgements

<b>Chapter 1 General Introduction</b>	<b>1</b>
1.1 Review of the Relevant Literature	1
1.1.1 General Cellular Actions of Aldosterone	2
1.1.2 Time-dependent Responses to Aldosterone	5
1.1.3 General Features of Transcellular Sodium Transport	6
1.1.4 Aldosterone Affects the Apical Entry of Sodium	9
1.1.4.1 Primary Hormonal Action: Increased Apical Conductance	11
1.1.5 Molecular Changes of Apical Membrane Conductance	11
1.1.5.1 Mechanisms of Changes to Channel Densities	15
1.2 Aims of the Study	20
<b>Chapter 2 Materials and Methods</b>	<b>24</b>
2.1 Cell Culture Methods	24
2.1.1 Selection of Inserts For Electrophysiological Experiments	25
2.2 Experimental Procedures	27

2.2.1	Tissue Treatment	27
2.2.1.1	Paired Tissues for Steady State Experiments	27
2.2.1.2	Tissues for Time Course Experiments	28
2.2.2	Electrophysiological Procedures	29
2.2.2.1	General Setup	29
2.2.2.2	Setup for Noise Analysis	30
2.2.2.3	Data Capturing and Processing	31
2.2.2.4	Blocker Induced Fluctuation Analysis	33
	Staircase Protocol for Steady State Experiment	34
	Pulse Inhibition Protocol for Time Course Experiments	36
<b>Chapter 3</b>	<b>Results</b>	<b>41</b>
3.1	Control Baseline Values	41
3.2	Effects of Aldosterone	45
3.2.1	Steady State Data: Late Aldosterone Effects	45
3.2.2	Pulse Inhibition Protocol Data: Earlier Aldosterone Effects	50

<b>Chapter 4 Discussion</b>	<b>57</b>
4.1 Control Baseline Values	57
4.2 Summary of the Steady State Data	58
4.2.1 Aldosterone Does Not Stimulate Channel Activity by Increases in Open Probability	58
4.2.2 Aldosterone Causes Large Increases in Channel Densities.	59
4.2.3 Speculations on the Reasons for the Differences in Results	61
4.2.3.1 Differences in Sodium Transport Rates	62
4.2.3.2 Are We Dealing with the Same Sodium channel?	64
4.2.3.3 Effects of Osmolarity	65
4.3 Comments on Possible Mechanism of Aldosterone Action on Sodium Transport	65
4.4 Conclusions	68
<b>Chapter 5 References</b>	<b>69</b>
<b>Appendix</b>	<b>79</b>

## Acknowledgements

The completion of this thesis would not have been possible without the tremendous support and encouragement I received from many others.

Firstly, I would like to sincerely thank my supervisor, Professor W. J. Els for his continuous support and guidance throughout my studies. For "Prof", no trouble was too great or too small and my vision was always quickly restored upon discussing any problem with him. He has been a great inspiration to me and I hope that I can carry throughout life, some of the principals he has taught me.

Secondly I would like to thank all the staff and students of the Anatomy and Cell Biology Department, each one of whom were an important link in the chain of support throughout my studies at U.C.T.. There were many chats and words of encouragement that all aided me in my completion of the study. I would like to acknowledge the technical assistance of Mr. Bruce Dando who always ensured that I received supplies on time, and Mr. Henry Fortuin for all his assistance.

Thirdly, I am deeply indebted to Paula Pieterse for her help especially with some of the diagrams that appear here. Her motivation and encouragement played a large part in the completion of this thesis.

Fourthly, a very special thankyou to my parents, Auriel and Paddy Baldwin for ensuring that I always have had the best opportunities in life. Thankyou too for your patience and advice throughout my studies.

Finally, I would like to acknowledge the financial assistance I received from The University of Cape Town (Frank Forman Award), the Physiological Society of Southern Africa (Cyril Wyndham Award) and the Foundation for Research and Development (FRD). In part this study was also funded by grants to Dr. W. J. Els from the Medical Research Council of South Africa (MRC) and the South African Kidney Foundation.

## Chapter 1

# 1 General Introduction

---

---

## 1.1 Review of the Relevant Literature

Epithelial sodium ( $\text{Na}^+$ ) transport is an essential process for homeostasis of body solute and water balance being, amongst others, the driving force for the absorption of water, sugars and other ions (Diamond, 1978). Accordingly, the regulation of epithelial  $\text{Na}^+$  transport is central to a wide variety of physiological processes (for review see Turnheim, 1994). The adrenal corticosteroid aldosterone, discovered in 1952 by Simpson *et al.*, is a major regulator of epithelial  $\text{Na}^+$  transport in vertebrates where its main target tissues are a group of epithelia collectively known as "tight epithelia"<sup>1</sup>. These epithelia include the cortical collecting duct (CCD), colon, salivary and sweat glands and certain amphibian epithelial model-tissues like the toad bladder and frog skin. In the mammalian nephron, the site of aldosterone action has been isolated, using stop flow analyses, to the distal nephron particularly the CCD where it stimulates the reabsorption of  $\text{Na}^+$  and the secretion of potassium ( $\text{K}^+$ ) and hydrogen ( $\text{H}^+$ ) ions (Sharp and Leaf, 1966; Schwartz and Burg, 1978).

A vast array of techniques have been employed to study  $\text{Na}^+$  transport and its regulation by hormones in many different target tissues. Much of the early work was performed on amphibian models including toad urinary bladder and frog skin. More recently, cultured cells have become popular model systems for the study of epithelial function as they offer the experimenter a large amount of material and the ability to strictly control the growing conditions (Handler *et al.*, 1980). Amongst these, A6 cells are a continuous epithelial cell

---

<sup>1</sup> "Tight epithelia" are characterized by a large transepithelial electrical potential difference and resistance (in the order of  $\text{k}\Omega\cdot\text{cm}^2$ ). The latter resides primarily in the tight junction, or zonula occludens, which encircles the epithelial cells near their apical surfaces, thus determining the resistance to ion flow in the paracellular pathway. The transcellular pathway is therefore the most significant, being responsible for regulated  $\text{Na}^+$  reabsorption even against steep electrochemical gradients.

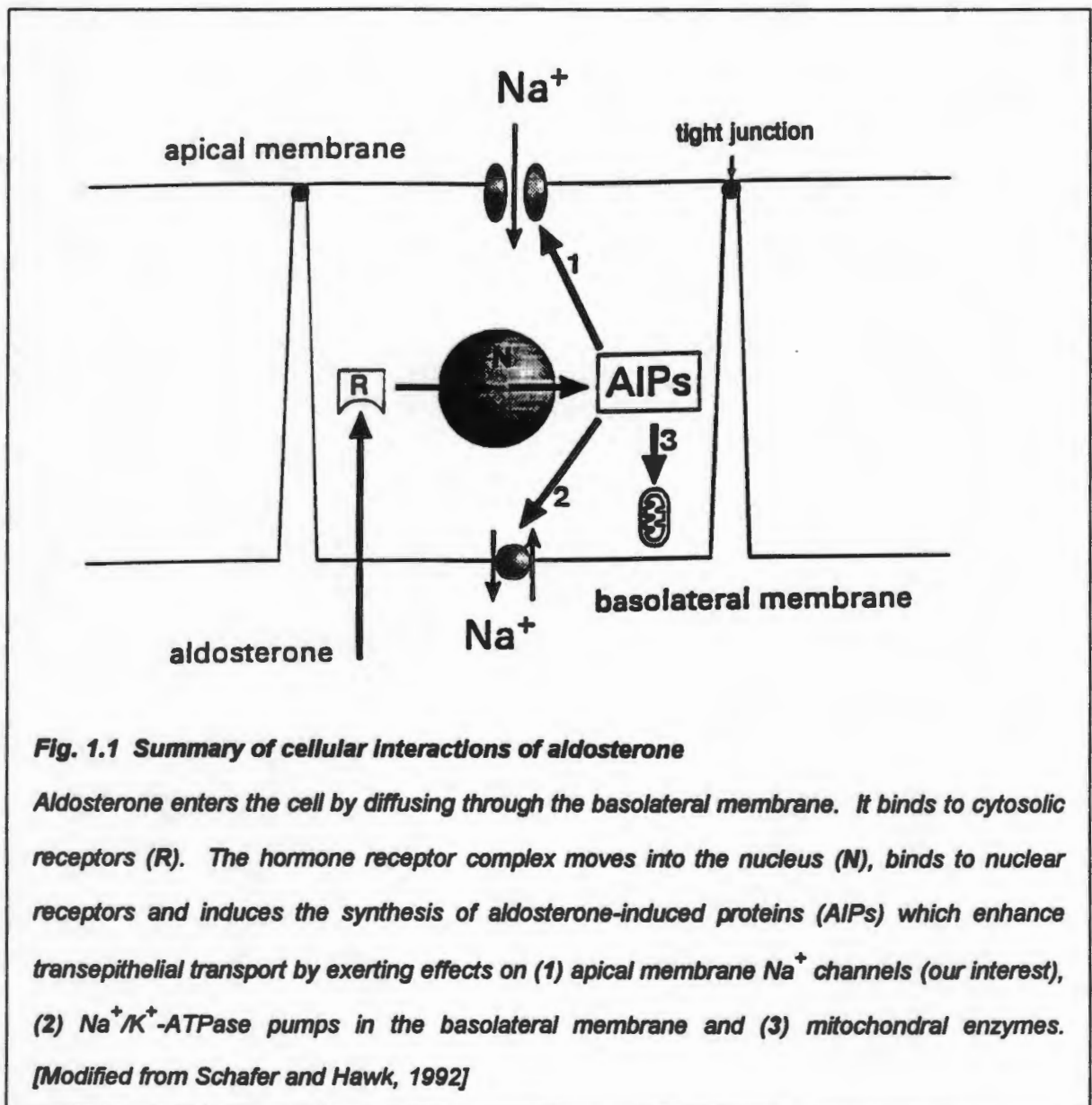
line derived from the distal nephron of the South African clawed toad (*Xenopus laevis*) (Rafferty, 1969). When cultured on permeable supports, these cells differentiate to form epithelial monolayers that transport  $\text{Na}^+$  and respond to aldosterone in a manner similar to mammalian CCD. Although the physiological effects of aldosterone have been known for about 40 years, the molecular mechanisms of its regulation of  $\text{Na}^+$  transport have yet to be fully resolved. Understanding these mechanisms is expected to also shed some light on clinically important disorders such as hypertension and other aldosterone related disorders (see White, 1994 and Rossier *et al.*, 1988).

### **1.1.1 General Cellular Actions of Aldosterone**

The importance of aldosterone as a regulator of ion transport was realised from studies carried out in the late 1950's. Live dogs, that were injected with aldosterone, showed reduced secretion of  $\text{Na}^+$  and increased secretion of  $\text{K}^+$  and  $\text{H}^+$  in the urine without a change in the glomerular filtration rate (Ganong and Murlow, 1958). In agreement,  $\text{Na}^+$  transport across frog skin could be enhanced with aldosterone and this effect could be blocked by the aldosterone antagonist, spironolactone (Cuthbert and Shum, 1975). Aldosterone is capable of stimulating  $\text{Na}^+$  transport over many hours. Obviously, during this time its effects will be determined by its metabolism within the cell, by the intracellular signalling systems that are activated by the hormone and also by processes that involve gene expression and protein synthesis (Lahav *et al.*, 1973). Hence, we start our introduction by presenting a brief overview of the cellular mechanisms of aldosterone.

The initial stages of the interaction of aldosterone with the cell may be summarised in the following way (Fig. 1.1). Being a steroid hormone, aldosterone is lipid soluble which allows it to enter the cell by diffusing across the plasma membrane and bind to its cytoplasmic receptors. Binding of the hormone ligand to its receptor, causes a conformational change which enables the hormone-receptor complex to move into the nucleus and bind to acceptor sites on chromatin. Here it regulates the transcription of messenger-ribonucleic acids

(mRNA) and the translation of a number of specific proteins termed the aldosterone-induced proteins (AIP's) which are responsible for inducing certain important physiological effects of the hormone (Edelman, 1981; Garty, 1986; Minuth *et al.*, 1987; Rossier *et al.*, 1988; Bastl and Hayslett, 1992 and Verrey, 1995).



**Fig. 1.1 Summary of cellular interactions of aldosterone**

*Aldosterone enters the cell by diffusing through the basolateral membrane. It binds to cytosolic receptors (R). The hormone receptor complex moves into the nucleus (N), binds to nuclear receptors and induces the synthesis of aldosterone-induced proteins (AIPs) which enhance transepithelial transport by exerting effects on (1) apical membrane Na<sup>+</sup> channels (our interest), (2) Na<sup>+</sup>/K<sup>+</sup>-ATPase pumps in the basolateral membrane and (3) mitochondrial enzymes.*

*[Modified from Schafer and Hawk, 1992]*

The induction of RNA synthesis by aldosterone was first reported by Rossier *et al.* (1974) who demonstrated that labeling of RNA with <sup>3</sup>H-uridine and <sup>3</sup>H-adenosine was enhanced during the first 30 minutes of hormonal action in toad bladder cells. Similar labeling experiments also revealed a higher incorporation of label into specific proteins after

stimulation with aldosterone. Evidence that the induction of mRNA does in fact initiate the transport response, comes from the observation of a linear relationship between the change in  $\text{Na}^+$  transport rate and the incorporation of radiolabelled amino acids after aldosterone exposure. The induction of RNA synthesis, in this case, was shown to be mineralocorticoid specific as enhanced label incorporation could not be induced by either glucocorticoids or the inactive 7- $\alpha$ -isoaldosterone and was blocked by spironolactone (Geheb *et al.*, 1981). Inhibiting RNA and protein synthesis with either actinomycin D or cycloheximide, also blocked the aldosterone-induced increase in the  $\text{Na}^+$  transport rate, indicating that the action of aldosterone is dependent on *de novo* protein synthesis (Edelman *et al.*, 1963; Chu and Edelman, 1972; Garty, 1986).

Aldosterone action involves the induction of AIPs but, it is still very unclear how these proteins bring about the physiological response to the transport rate. Towards this, many studies have been dedicated to isolating and identifying AIPs. It appears that AIPs isolated so far, can be divided into three different groups: 1) AIPs with unknown functions, 2) subunits of the  $\text{Na}^+/\text{K}^+$ -adenosine triphosphatase (ATPase) pumps and 3) mitochondrial enzymes such as citrate synthase and flavokinase (Minuth *et al.*, 1987). While the latter two groups play vital roles in the overall stimulation of  $\text{Na}^+$  reabsorption, it is the proteins belonging to the first group, with as yet unknown function, that are thought to be involved in initiating the natriuretic effect of aldosterone. By employing two-dimensional gel electrophoresis and autoradiography, Cox and co-workers identified a group of 4 to 6 membrane bound glycoproteins (molecular weight  $M_r$  65 to 80 kD) in toad urinary bladder (Geheb *et al.*, 1981) and A6 cells (Blazer-Yost *et al.*, 1982) that are induced by aldosterone. Szerlip *et al.* (1989) raised monoclonal antibodies to these proteins and localised, with the aid of light and electron immunohistochemistry, the 70 kD glycoprotein (GP) to the apical membrane and subapical granules. Interestingly, the isolated and purified bovine  $\text{Na}^+$  channel ( $M_r$  730 kD) has been resolved into 5 polypeptides with  $M_r$ : 315, 149, 95, 71, and 55 kD, while a few minor polypeptides ( $M_r$  30-45 kD) were often observed (Benos *et al.*,

1987 and for reviews see Garty and Benos, 1988; Sariban-Sohraby and Fischer, 1995). GP 70 shows cross-reactivity with the 70 kD subunit of the purified bovine Na<sup>+</sup> channel protein in Western blots, raising the possibility that GP 70 may represent a component or modulator of the Na<sup>+</sup> channel.

### **1.1.2 Time-dependent Responses to Aldosterone**

Since the regulation of membrane Na<sup>+</sup> conductance depends on protein synthesis, it stands to reason that there would be important time-dependent changes in the mechanisms involved in the stimulation of transport. In earlier initial studies where dogs were injected with aldosterone, changes in their urine composition were only seen after a delay of 30 to 60 minutes, even when aldosterone was injected directly into the renal artery (Ganong and Murlow, 1958). Similar delays in the physiological responses of the hormone were also observed in a number of other ion transporting epithelia. Accordingly, perusal of the literature shows that the transport response of "tight epithelia" to aldosterone can be divided into 2 phases. The first period is termed the latent phase which lasts anything between 30 to 120 minutes during which time, there is little or no change to the  $I_{sc}$ . It is thought that the aldosterone-induced protein synthesis, necessary for the transport response, occurs during this period. The second phase that follows the latent phase is characterised by a 2 to 5 fold increase in the  $I_{sc}$  and, hence, is referred to as the natriferic response. The natriferic response to the hormone has been further divided into early and late phases (see Spooner and Edelman, 1975; Rossier *et al.*, 1985; Garty, 1986). However, the physiological response to Na<sup>+</sup> transport may vary widely among different epithelia in terms of the magnitude of response and duration of each phase (review by Shafer and Hawk, 1992). Further, many different factors could affect the baseline rate of Na<sup>+</sup> transport, including, circulating levels of hormones in live specimens, foetal calf serum content in cultured cells and osmolarity of the culture medium.

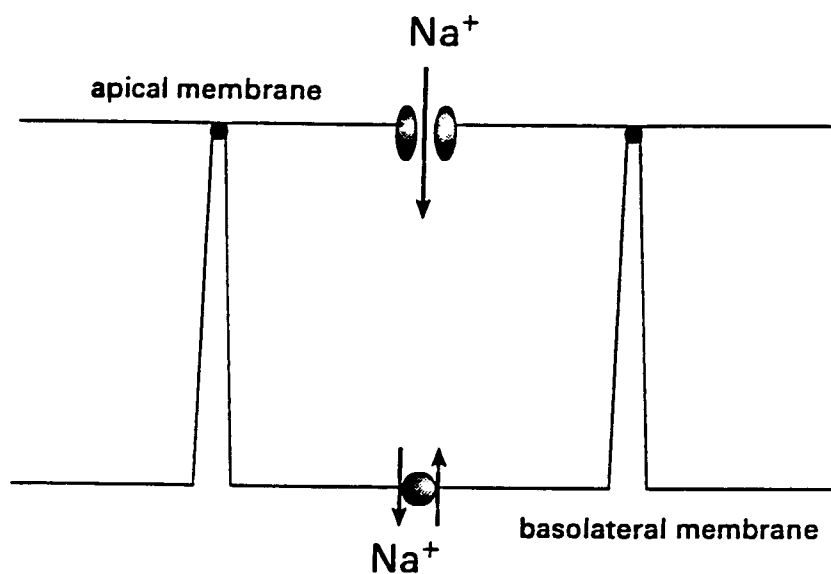
The natriuretic-response phase induced by aldosterone is characterised by an increase in the short circuit current ( $I_{sc}$ ) which, especially during the early phase, is accompanied by an increase in apical membrane conductance (Wills *et al.*, 1993; reviews by Garty, 1986; Minuth *et al.*, 1987; Schafer and Hawk, 1992; Bastl and Hayslett, 1992; Verrey 1995). The increases in the transport rate that occur during the late natriuretic phase are more complex and may or may not involve changes in membrane conductance, but are accompanied by increased activity of the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase. Hence, the entry and extrusion rates of  $\text{Na}^+$  are both enhanced (review by Verrey, 1995). However, an increase in apical membrane conductance during the late natriuretic response may be of greater importance in epithelia, such as A6 cells, that exhibit comparatively low baseline levels of  $\text{Na}^+$  transport (Verrey, 1994).

### **1.1.3 General Features of Transcellular Sodium Transport**

Understanding the manner in which  $\text{Na}^+$  is reabsorbed across an epithelium is intimately associated with its structure. Hence, the expression of epithelial cell surface proteins in a polarised manner is essential for the vectorial transport of solutes and the secretion of solutes and proteins.  $\text{Na}^+$  transporters and channels tend to be concentrated at the apical pole, whereas  $\text{Na}^+/\text{K}^+$ -ATPase pumps, along with the mitochondria that provide energy to drive the pumps, tend to be localised towards the basolateral membrane. Tight junctions ensure that this polarity in membrane composition is maintained by blocking, to any great extent, the diffusion of proteins between the two membrane regions (Barry, 1987).

Our understanding of transepithelial  $\text{Na}^+$  transport and its regulation by hormones, is based on a two step model proposed by Koefoed-Johnson and Ussing in 1958 to describe the  $\text{Na}^+$  transport across frog skin. This model has been adapted to describe the process across "tight epithelia" in general (Fig. 1.2). In the first step,  $\text{Na}^+$  ions diffuse passively down an electrochemical gradient and into the cell via an amiloride-blockable pathway in the apical membrane. The concept of how  $\text{Na}^+$  crosses the apical membrane perplexed scientists for

many years. Initially, most of the available data apparently favoured the idea of protein transporters, until Lindemann and Van Driessche (1977), using a modified method of fluctuation analysis (blocker-induced noise analysis), demonstrated for the first time that the rate of transport across the apical membrane ( $10^6$  ions/sec) was about 100 fold too fast for a transporter. This was the first direct evidence for the existence of  $\text{Na}^+$  channels, through which  $\text{Na}^+$  is able to enter into cells, in the apical membranes of tight epithelia. Such channels were subsequently also confirmed by patch clamp studies on native renal epithelia (Helman *et al.*, 1985; Palmer and Frindt, 1986a, 1986b) and in cultured A6 cells (Hamilton and Eaton, 1985). The second step involves the active extrusion of  $\text{Na}^+$  across the basolateral membrane by  $\text{Na}^+/\text{K}^+$ -ATPase pumps which serves to keep the intracellular sodium concentration,  $[\text{Na}^+]_i$ , at a low level, thereby maintaining the electrochemical gradient necessary for passive  $\text{Na}^+$  entry into the cell.



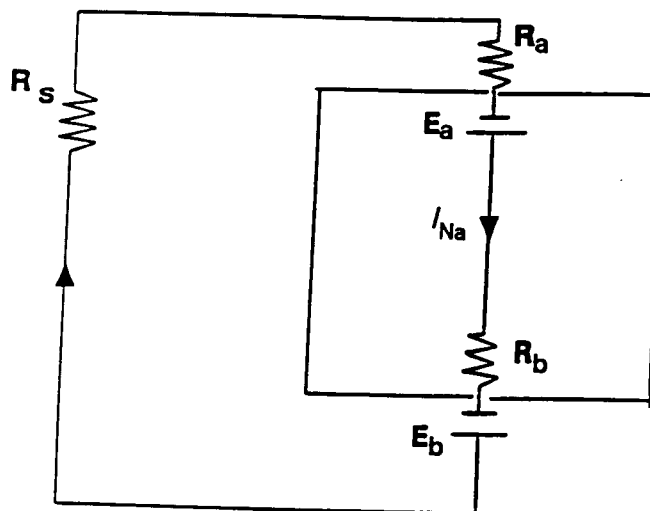
**Fig. 1.2 Model of transepithelial  $\text{Na}^+$  transport**

$\text{Na}^+$  enters the cell, down its electrochemical gradient, through  $\text{Na}^+$  channels in the apical membrane and is actively extruded via  $\text{Na}^+/\text{K}^+$ -ATPase pumps in the basolateral membrane, constituting the transcellular pathway. Tight junctions (•) near the apical surface determine the conductance of the paracellular pathway. [Consistent with Koefoed-Johnsen and Ussing (1958)].

Ussing and Zerahn (1951) proposed an electrical model to describe the important features of the  $\text{Na}^+$  transport process across frog skin, based on a Thévenin emf ( $E_{\text{Na}}$ ) in series with a Thévenin resistance to  $\text{Na}^+$  transport ( $R_{\text{Na}}$ ). This model was later expanded by Helman (1979) to include further Thévenin equivalents at both the apical, (subscript "a") and basolateral, (subscript "b") membranes such that:

$$E_{\text{Na}} = E_a + E_b \text{ and } R_{\text{Na}} = R_a + R_b$$

In addition to the transcellular route, ions may also flow through the paracellular pathway, between cells. The resistance to ion flow through this pathway (determined mainly by the properties of the tight junctions) can be modeled by the shunt resistance ( $R_s$ ) (Fig. 1.3).



**Fig. 1.3** Thévenin electrical equivalent circuit model of transepithelial  $\text{Na}^+$  transport

$R_a$  and  $R_b$  indicate the resistance to transcellular  $\text{Na}^+$  transport at the apical and basolateral membranes, respectively. The paracellular pathway, which is in parallel with the transcellular pathway, is characterised by the shunt resistance,  $R_s$ . Transcellular  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) is generated by unequal electrochemical diffusion potentials ( $E_a$  and  $E_b$ ) at the apical and basolateral membranes respectively. Overall transepithelial voltage ( $E_T$ ) is usually apical side negative with respect to the basolateral side. [Adapted from Helman and Fisher, 1977].

In the presence of a low shunt resistance, ions can pass through this circuit via both the transcellular and the paracellular route. The current through the paracellular pathway can be negated by voltage clamping the transepithelial potential to zero. The resultant short circuit current ( $I_{SC}$ ), in tight epithelia, is usually a good measure of the  $\text{Na}^+$  transport rate, the  $I_{\text{Na}}$  (Ussing and Zerahn, 1951). Many of the subsequent studies on  $\text{Na}^+$  transport are based on the principles inherent in these electrical models.

#### **1.1.4 Aldosterone Affects the Apical Entry of Sodium**

From the preceding discussion, it is clear that a hormone could generally cause a change in transepithelial  $\text{Na}^+$  transport by affecting either the entry rate of  $\text{Na}^+$  through apical  $\text{Na}^+$  channels or the extrusion rate of  $\text{Na}^+$  via the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase pumps. As shown in Fig. 1.1, the situation for the mechanisms of aldosterone's actions is complicated by the fact that it could also modulate the rate of  $\text{Na}^+$  transport through effects on the metabolic pathways.

On the evidence that the  $[\text{Na}^+]_i$  does not change, even at the height of the aldosterone-induced  $\text{Na}^+$  transport response, Lipton and Edelman (1971) proposed that aldosterone has co-ordinated effects at both membranes, increasing entry and extrusion of  $\text{Na}^+$  and that both effects are mediated by an enhanced energy supply. While it is well known and appreciated that aldosterone does have effects at the basolateral membrane, and keeping in mind that it does have effects on the metabolic pathways of the cell, our main interest for this study centres on effects at the apical membrane. The reason for this shall become evident during the following discussions.

Much of the early work focused on identifying the main cellular site of action of aldosterone. It was obvious to experimenters at the time that if a rate limiting step in the transport of  $\text{Na}^+$  could be identified, that particular step would be a likely target of a hormone involved in regulating  $\text{Na}^+$  reabsorption. Crabbé (1961) reported the first in vitro stimulation of  $\text{Na}^+$

reabsorption by aldosterone in the toad urinary bladder. He demonstrated that aldosterone caused an increase in the  $I_{sc}$  after about an hour, which was accompanied by an increase in transepithelial membrane voltage. These results, supported by the observation that aldosterone elevated a  $^{22}\text{Na}^+$  isotope flux across the tissues, suggested that a main effect of aldosterone is exerted on a rate limiting step, probably at the apical membrane. Sharp and Leaf (1966) also suggested that the entry step through the apical membrane was rate limiting after demonstrating that the entry of  $\text{Na}^+$  ions could be saturated. Direct proof, though, had to await results from electrical studies using intracellular microelectrodes which allowed the measurement of electrochemical driving forces and membrane conductances for ion transport across separate membranes. In such initial studies on toad urinary bladder (Civan and Hoffman, 1971) and on frog skin (Saito and Essig, 1973), it was shown that aldosterone stimulates the  $\text{Na}^+$  transport rate by increases to the transepithelial conductance ( $G_T$ ), mainly through enhancement in the conductance of the transcellular pathway ( $G_t$ ). Although one study on A6 cells suggests an increase in the paracellular conductance ( $G_j$ ) (Fidelman and Watlington, 1987), a later study also on A6 cells verified that the main effect of aldosterone is to increase  $G_t$  and not  $G_j$  (Wills *et al.*, 1993). Leaf and MacKnight (1972) provided additional evidence that the rate limiting step in  $\text{Na}^+$  reabsorption might be located at the apical membrane, after they demonstrated an increased intracellular pool of radiolabelled,  $^{22}\text{Na}^+$  (initially added to the mucosal solution) after stimulation of toad urinary bladder with aldosterone. Further, microelectrode studies on isolated perfused rabbit CCD revealed that the site of lowest transcellular conductance is in fact the apical membrane, and treatment with aldosterone for 11 to 18 days caused an expected increase in the voltage across the apical membrane from 8.5 mV to 58.2 mV (Helman, 1973). So, the evidence presented above showed clearly that the entry of  $\text{Na}^+$  ions across the apical membrane represents the rate limiting step in the reabsorption of  $\text{Na}^+$  and, accordingly, a likely important target in the action aldosterone.

#### **1.1.4.1 Primary Hormonal Action: Increased Apical Conductance**

As we have seen, aldosterone causes an increase in  $\text{Na}^+$  transport by increasing the transepithelial conductance to  $\text{Na}^+$ . This could possibly occur by a primary hormonal effect on the apical membrane  $\text{Na}^+$  permeability or conductance ( $G_a$ ) or secondary to an increased driving force for  $\text{Na}^+$  entry ( $E_{\text{Na}}$ ) due to an enhanced pump rate. However, in studies on toad bladder, Hong and Essig (1976) were able to demonstrate that aldosterone does not affect the ( $E_{\text{Na}}$ ). Nagel and Crabbé (1980) provided the first conclusive proof that a main effect of aldosterone on  $\text{Na}^+$  transport is to induce an increase in  $G_a$ . With microelectrode studies on toad skin, they determined that aldosterone causes a decrease in apical membrane resistance (or an increase in  $G_a$ ). Results from several studies have since confirmed the suggestion that aldosterone increases  $G_a$ , both by electrical measurements in intact epithelia (Palmer *et al.*, 1982, Lewis and Wills, 1983 and Sansom and O'Neil, 1985) and by measuring  $\text{Na}^+$  fluxes into isolated apical membrane vesicles (Sariban-Sohraby *et al.*, 1984a; Asher and Garty, 1988).

#### **1.1.5 Molecular Changes of Apical Membrane Conductance**

The development of particularly the new electrophysiological techniques have provided a means of studying the mechanisms whereby aldosterone may exert its effects on the  $\text{Na}^+$  channels in the apical membranes of renal and other epithelial cells. Several quantitative electrical studies on a variety of epithelia have shown that the apical membrane conductance can be linearly correlated with changes to the  $I_{\text{sc}}$  caused by aldosterone (Palmer *et al.*, 1982, Garty *et al.*, 1983, Nagel and Katz, 1991 and Turnheim, 1994). These results, consistent with the data from earlier studies by Lindeman and Van Driessche (1977) and confirmed by flux studies into isolated apical membrane vesicles (Sariban-Sohraby *et al.*, 1984), supported the idea that aldosterone exerts its natriuretic effects mainly by modulating  $\text{Na}^+$  channels in the apical membrane.

Conceptually, there are a number of possible mechanisms whereby aldosterone could cause modulatory changes to the  $\text{Na}^+$  channels which would result in an increase in the  $\text{Na}^+$  conductance of the apical membrane, responsible for the increase in  $\text{Na}^+$  transport. In general, the magnitude of  $G_a$  depends upon three parameters of the  $\text{Na}^+$  channels, outlined by the following relationship:

$$G_a \propto \gamma_{\text{Na}} \cdot P_o \cdot N_o$$

where,  $\gamma_{\text{Na}}$  is the single channel conductance,  $P_o$  is the open probability of a single channel and  $N_o$  is the number of open  $\text{Na}^+$  channels per unit area of membrane, at any given moment. In theory, changing any one of the variables on the right hand side must alter the apical membrane conductance which in turn governs the magnitude of current through a group of channels in the apical membrane.

In general, hormones do not affect the conductance of individual channels but exert their effects by changing either the  $P_o$  or the number of active channels (review by EIs and Helman, 1989). This apparently also applies to the mechanisms of action of aldosterone since there is little, if any, support for the idea that aldosterone may alter the channel conductance of individual  $\text{Na}^+$  channels. The mechanisms whereby aldosterone modulates the gating of apical membrane  $\text{Na}^+$  channels is not clear and, as we shall see, recent results have generated an ardently debated controversy regarding which parameters are mainly affected by aldosterone in its stimulation of the  $\text{Na}^+$  transport rate. Given that the hormone apparently does not alter  $\text{Na}^+$  channel conductance, its main effects must be to modulate either or both of the remaining variables, namely channel open probability and channel density. There is convincing evidence that the main effect of aldosterone is to bring about large changes in apical membrane channel densities (reviewed by Garty, 1986). However, as we shall see, the mechanisms are probably complex and could well involve multiple regulatory mechanisms.

Arguing against the view that aldosterone causes an increase in the number of  $\text{Na}^+$  channels, are results with patch clamp studies on A6 cells (Kemendy *et al.*, 1992). The readdition of aldosterone to cells depleted of the hormone for 48 h caused marked alterations to the kinetics of  $\text{Na}^+$  channels. Specifically, and under these conditions, aldosterone caused near 35-fold increases to the mean open time and near 10-fold increases to the channel  $P_o$  from values near zero. As a result, they suggested that one major mechanism whereby aldosterone alters  $\text{Na}^+$  transport is through changes to  $P_o$ . Although the readdition of aldosterone did increase the number of current levels across the patch (and may contribute to the increase in  $\text{Na}^+$  transport), this increase was reported as not statistically significant and it was concluded that there was no change in channel number. Other measurements by Kleyman *et al.*, (1989) with an antibody to the amiloride binding site of  $\text{Na}^+$  channels and a photoactive amiloride analogue have demonstrated that the number of  $\text{Na}^+$  channel proteins are not significantly altered by aldosterone within 16 h, suggesting that aldosterone does not have a main effect on the regulation of  $\text{Na}^+$  channel densities during this time.

The idea that aldosterone stimulates  $\text{Na}^+$  transport by increases in apical membrane channel density was suggested earlier by Frizzell and Schultz (1978). Their experiments were based on the observations that amphotericin B produced an increase in  $\text{Na}^+$  reabsorption, very similar to those induced by aldosterone, by shunting (inducing pores in) the apical membrane (Crabbé, 1967). Although pre-treatment with aldosterone caused a doubling of the  $\text{Na}^+$  influx across rabbit colon, this effect could not be enhanced further by additional administration of amphotericin B and *vice versa*. Hence, it was postulated that aldosterone increases  $\text{Na}^+$  transport concurrent with increases to the number of conducting channels. Similar actions have been proposed for the action of aldosterone on renal and renal-model tissues based on channel labelling and electrophysiological techniques. It was shown in toad bladder, that aldosterone doubled the number of  $^{14}\text{C}$ -amiloride binding sites after 4 hours (Cuthbert and Shum, 1975). This however, could have been a secondary

response due to changes in intracellular  $\text{Na}^+$  concentration or membrane potential (Cuthbert and Shum, 1977). The advent of fluctuation analysis made it possible to examine the effects of aldosterone on the number of open channels more directly. Results from such studies strongly suggest that mineralocorticoids (like aldosterone) and even glucocorticoids (such as dexamethasone), may stimulate  $\text{Na}^+$  transport mainly by increases to  $\text{Na}^+$  channel densities, without an apparent effect on the kinetics of the channels (Palmer *et al.*, 1982; Fischer and Clauss 1990; Granitzer *et al.*, 1995). This idea was also supported by patch clamp data (Palmer and Frindt, 1986a, 1986b). Plasma aldosterone levels were elevated in rats by placing them on a low  $\text{Na}^+$  diet for 12 h. This manoeuvre caused an increase to the mean number of open channels per patch in the rat CCD, compared to control rats that had not been placed on the low- $\text{Na}^+$  diet. There was no change to the  $P_o$  of  $\text{Na}^+$  channels, suggesting that, contrary to the conclusion by Kemendy *et al.* (1992), aldosterone may mainly regulate  $\text{Na}^+$  channel densities. In further very similarly designed experiments, Frindt *et al.*, 1990 reported that the number of active channels increased from about 100 per cell in rats on the normal  $\text{Na}^+$  diet to 3000 per cell in rats on the low  $\text{Na}^+$  diet. Other results, apparently consistent with this idea, came from impedance data and from preliminary studies with noise analysis, respectively (Wills *et al.*, 1993; Baxendale *et al.*, 1987). Wills *et al.* (1993) observed that the conductance of the apical membrane was significantly increased for aldosterone-treated epithelia compared to aldosterone depleted controls. Apical membrane capacitance was not affected which is consistent with a higher density (number of channel per membrane area) of conducting  $\text{Na}^+$  channels in this membrane following aldosterone stimulation.

### 1.1.5.1 Mechanisms of Changes to Channel Densities

The mechanisms whereby changes to channel densities are produced by hormones in general and, by aldosterone in particular, has perplexed investigators for many years and still remain unclear. By consensus, it seems that aldosterone may induce changes to channel densities by one or more of the following mechanisms: (1) the *de novo* synthesis and subsequent insertion of newly formed channels, (2) by the activation of non-conductive channels or, (3) by a dynamic exchange between cytoplasmic and membrane pools of channels (reviews by Palmer and Sackin, 1988; Bradbury and Bridges, 1994). These schemes could also involve time-dependent mechanisms.

In the light of the dependence of the transport response on protein synthesis, the synthesis and/or insertion of *de novo* channel proteins is most probably somehow involved in this scheme. Studies by Kleyman *et al.* (1989) demonstrated that 16 h after the addition of aldosterone to A6 cells, there were no changes to the pool of cellular channels, suggesting that within this time frame at least (consistent with the latent and earlier natriuretic periods of the response), responses to the hormone do not involve channel synthesis. Subsequently, it was shown that RNA isolated from toad bladder was able to express functional channels cells in frog oocytes only after being treated with aldosterone for 20 h, suggesting that the late phase of the natriuretic action could in part be the result of the synthesis of new channels (Asher *et al.*, 1992).

The bulk of the data on the mechanisms of action of aldosterone seemingly favour the idea that channel densities may be regulated by direct activation. Data from careful and extensive studies by Garty and Edelman (1983) on toad urinary bladder epithelium strongly supported this idea. Epithelial Na<sup>+</sup> channels are sensitive to proteolysis by trypsin. When trypsin was applied to the surface of toad bladder cells, it blocked the subsequent aldosterone-dependent increase in the Na<sup>+</sup> transport (at least to the same extent as it blocks the baseline transport). These results suggest that the aldosterone-induced

stimulation of the  $I_{Na}$  involves channel proteins present in the membrane and, hence, support the idea of channel activation. There were concerns that the non-specific action of trypsin may cause the lysis of other membrane proteins which may be necessary for the insertion of channels into the membrane. Evidence against this concern is two fold: Firstly, although pre-trypsinisation of the apical membrane reduced the aldosterone-induced increase in the  $I_{SC}$ , the response to aldosterone was not completely blocked (approximately to the same degree as the depletion of the baseline  $I_{SC}$ ) and its time course of action was not altered. This suggests that, although reduced, some of the prerequisites for the stimulation by aldosterone are in fact still preserved after trypsin proteolysis. Secondly, amiloride (30  $\mu$ M) added to the apical solution before exposure to trypsin, could preserve both the baseline and aldosterone-dependent  $I_{SC}$ , whereas  $Na^+$ -free Ringer's solution did not have the same protective effect.

There might well be different mechanisms of aldosterone action responsible for the increase in  $Na^+$  transport rate during the different phases of the response. Sariban-Sohraby *et al.* (1984) reported that short-term exposure to aldosterone increased the  $^{22}Na^+$  flux into isolated A6 membrane vesicles, but that neither the total yield of vesicle protein, nor an apical membrane marker ( $\gamma$ -glutamyltranspeptidase) were elevated, suggesting that channels affected by the hormone are already in the membrane. Additional, though indirect, evidence supporting the activation theory was also presented by data that indicated that the short-term addition of aldosterone (3 h) increased channel activity in toad bladder epithelium but, that the RNA from those cells were unable to express functional channels in the frog oocyte (Asher *et al.*, 1992). This result argues against the insertion or recruitment theory, during especially the early response to aldosterone.

One way in which channels may respond to direct activation is by increasing the channel open probability. We have already alluded above to the fact that there is uncertainty regarding the manner in which aldosterone may alter the gating of  $Na^+$  channels. While

results by Kemendy *et al.* (1992) seemingly favour the idea that aldosterone increases the  $P_o$  of apical channels, not all data is consistent with this idea. How channels may be directly activated remains ambiguous, but recent studies examining this very question have proposed some interesting ideas.

Results from recent studies have shown that aldosterone may act by modulating a variety of biochemical reactions which may result in the direct activation of  $\text{Na}^+$  channels. Amongst these the enhanced metabolism of fatty acids by a stimulation of the activity of phospholipase A, the methylation of apical proteins and lipids and the control of carboxymethylations by G proteins may play a role (Garty, 1986; Minuth *et al.*, 1987; Sariban-Sohraby and Fisher, 1995; Verrey, 1995). Although outside the scope of this study, it is of interest (also for future research projects in our laboratory) to briefly review the possible mechanisms whereby the gating of channels may be regulated. Regarding these, the possible roles of especially aldosterone-induced methylation in channel activation have recently received much attention (reviewed by Sariban-Sohraby and Fisher, 1995). The first indication that a methylation step might be involved came from studies on cultured toad bladder cells (Wiesman *et al.*, 1983) where aldosterone increased the incorporation of radioactively labelled methyl groups into proteins and phospholipids. Later, Sariban-Sohraby *et al.* (1984b) showed that a 30 minute incubation with the methyl donor, S-adenosylmethionine (SAM) caused a doubling in the  $^{22}\text{Na}^+$  flux into apical membrane vesicles isolated from cultured A6 epithelia. A similar increase in amiloride-sensitive  $\text{Na}^+$  transport and methylation of proteins and phospholipids was observed when vesicles were exposed to aldosterone for 5 hours. However, in vesicles from cells pre-treated with aldosterone, the intracellular  $^{22}\text{Na}^+$  was already elevated and could not be augmented with the subsequent addition of SAM. These results were interpreted as indicating that aldosterone increases transport across the apical membrane by methylating proteins and/or phospholipids and, further, that when the proteins were already methylated by prior exposure to aldosterone, there could be no further increase in transport rate by SAM.

Patch clamp studies also indicated that the increase in channel activity caused by aldosterone could be blocked by inhibiting the methylation process with the inhibitor 3-deazaadenosine (Kemendy and Eaton, 1990). Sariban-Sohraby *et al.* (1993) reported that a 90 kD polypeptide of the isolated Na<sup>+</sup> channel is a target of aldosterone-induced carboxymethylation in intact A6 epithelia and in apical membrane vesicles derived from A6 cells.

Since only the short term effects (3 to 6 hours) of aldosterone were analysed in the trypsin and other experiments reviewed above, the possibility remains that at least the longer term effects of aldosterone may involve the synthesis and/or insertion of new channel proteins. In fact, the reversible transfer of channels from cytoplasmic stores to the apical membrane may well be an important mechanism whereby hormones and other factors regulate apical membrane permeability through changes in channel densities. This mechanism, suggested by Lewis and de Moura (1984) is possibly responsible for the increase in Na<sup>+</sup> channel densities during autoregulatory changes to apical membrane conductance in frog skin (Els and Chou, 1993). It is also the mechanism whereby insulin increases the number of glucose transporters in membranes (Tsakiridis *et al.*, 1994) and whereby antidiuretic hormone increases the water permeability of the medullary CD (review by Handler, 1988). In renal epithelia, stimulation of the Na<sup>+</sup> transport rate by vasopressin may occur by the recruitment of channels from the cytoplasm (Garty and Edelman, 1983; Schafer and Hawk, 1992; Chou and Els, 1996 submitted). Whether aldosterone makes use of the same mechanism to increase channel density is undecided, but it remains a distinct possibility. The idea that changes in channel densities, responsible for alterations to ion and solute transport, may be regulated by a controlled recycling of subapical and cytoplasmic vesicles is an exciting development and has recently been reviewed (Bradbury and Bridges, 1994).

The current data seem to favour the idea that most probably the two major hormones in control of Na<sup>+</sup> reabsorption, vasopressin (AVP) and aldosterone, do so by different

mechanisms. Obviously, newly synthesised channels have to be inserted but, whether by an aldosterone-regulated process is not known. Immunocytochemical data should provide the most compelling evidence for or against the insertion theory. Results using a double labelling method with an amiloride analogue and anti-amiloride antibodies, showed that the total pool of  $\text{Na}^+$  channels was not altered after exposure to 300 nM aldosterone for 16 hours, despite a 4 fold increase in the  $I_{\text{sc}}$  and a doubling of the apical conductance (Kleyman et al., 1989), data which may be interpreted as arguing against the insertion theory (at least for the early response). This assumption was also supported by results with polyclonal antibodies against the  $\text{Na}^+$  channel that seemingly indicated that aldosterone did not stimulate the fusion of vesicles with the apical membrane of transport epithelia (Tousson *et al.*, 1989). However, it is important to point out that interpretation of such experiments can be difficult since amiloride is known to bind to all channels in the active pool (in open and closed states) and so it is feasible that it could also bind to non-conducting channels, while, in addition, the amiloride molecule may have multiple binding sites at the  $\text{Na}^+$  channel. Hence, the specificity of current immunocytochemical studies are questionable.

Capacitance measurements of apical membrane area may provide another slant on the idea of vesicle insertion. Using impedance analysis, Wills et al. (1993) reported a large increase in the conductance of the apical membrane of A6 cells, but without any effect on the membrane capacitance following exposure to aldosterone for less than 4 hours. These data support the conclusions of the earlier trypsin experiments, suggesting that the early effects of aldosterone do not occur by an increase in the total pool of channels. In contrast, recent impedance analysis on A6 cells have shown an increase in the surface area of the apical membrane after exposure to aldosterone for 18 to 24 hours (Liu *et al.*, 1995). These results seemingly suggest that during at least the late natriuretic response, effects of aldosterone may also involve an insertion of newly synthesised channels into the apical membrane.

## 1.2 Aims of the Study

Aldosterone is an important hormone responsible for maintaining  $\text{Na}^+$  homeostasis in vertebrates. In tight epithelia such as the CCD, rabbit distal colon and toad bladder, addition of the hormone markedly increases the rate of  $\text{Na}^+$  transport through increases of apical membrane  $\text{Na}^+$  permeability over a period of many hours. Vectorial  $\text{Na}^+$  transport across these epithelial cells is mediated by amiloride-sensitive  $\text{Na}^+$  channels in the apical membrane and by the  $\text{Na}^+/\text{K}^+$ -ATPase at the basolateral membrane. With the advent of the new electrophysiological techniques, it has been demonstrated quite clearly that aldosterone has important regulatory effects on the apical membrane  $\text{Na}^+$  channels. Despite a significant number of investigations, the precise modulatory effects that the hormone has on the apical channels and whereby it increases membrane permeability are unresolved.

The results from earlier studies by mainly Palmer and his colleagues suggested that, consistent with the action of AVP (another hormone with strong natriuretic effects), the main action of aldosterone is to increase the number of  $\text{Na}^+$  channels concurrent with an increase in apical membrane permeability. The studies were performed with amiloride-induced fluctuation analysis (Palmer *et al.*, 1982) and the hypothesis was supported by later results from patch clamp studies, supplemented by membrane capacitance measurements (Frindt *et al.*, 1990; Pácha *et al.*, 1993). However, the actions of aldosterone on the principal cells of the CCD were only examined indirectly, on rats that were maintained on a low  $\text{Na}^+$  diet to elevate plasma levels of aldosterone. Moreover, results from immunocytochemical studies were unable to support this hypothesis since they found no evidence that aldosterone causes an increase to the pool of presumptive  $\text{Na}^+$  channels (Kleyman *et al.*, 1989). In a more recent study using the patch clamp technique, the direct effects of aldosterone were studied in detail on cultured A6 cells by Kemendy *et al.* (1992). Based on their results, the investigators suggested that contrary to the other

studies, the main action of aldosterone is to induce very large increases in the channel open probability, due to an increase in the mean open time, without any significant effect on the number of channels.

These contradictory results are unfortunately clouding important issues regarding our understanding the mechanisms of action of aldosterone in the control of  $\text{Na}^+$  homeostasis. For academic and scientific persuasions, it is important that clarity and consensus should be reached about its main mechanisms of action. We already find that in recent reviews it is suggested that the main function of aldosterone is to increase  $P_o$  of the channels and that, therefore, aldosterone and AVP stimulate  $\text{Na}^+$  transport by different mechanisms (c.f. Schafer and Hawk, 1992; Eaton *et al.*, 1995). This may be the correct assumption, but since there is increasing evidence from other sources that are not consistent with the latter hypothesis, we devised experiments to specifically investigate whether aldosterone has main effects on the  $P_o$  and/or on the total number of channels in the apical membranes of a tight epithelium.

In the design of our experiments we took cognisance of the following issues. The most important issue here is to distinguish between changes in channel density and channel open probability. This is not so easy to do and most often this question is addressed by studies performed with either patch clamp or fluctuation analysis. The advantages and disadvantages of each method have been reviewed in an edition on current methods in electrophysiological techniques to which, major exponents of each method have contributed (Helman and Van Driessche, 1990). With patch clamp, one is really only investigating the area of membrane under the patch pipette and therefore one has no idea of the events in the remainder of the membrane. What is actually measured is the number of channels open beneath the pipette, or the channel "activity" ( $NP_o$ ). This technique does not measure the total number of channels (open and closed, in the whole membrane). Also, one cannot short-circuit the cell under patch-clamp conditions and hence valuable macroscopic data is

unavailable. The method is also sometimes invasive, especially when studying excised patches and may disrupt the normal physiology of the membrane (Ruknudin *et al.*, 1991). In some cases, treatment of the membrane with proteolytic enzymes such as trypsin may be necessary to remove certain glycoproteins in order to obtain a high resistance seal (Wills and Zweifach, 1987) and this may have grave effects on physiological functioning of the channel and on the integrity of the membrane. Patch clamp is still the best method to study single channel kinetics. On the other hand, fluctuation analysis provides information regarding the channels only indirectly and the method is based on a number of assumptions regarding the channels. Previously there has been some concern regarding the calculation of the channel corner frequency ( $f_c$ ) and the contribution of  $1/f$  noise to the measurements (Kemendy *et al.*, 1992). These are valid concerns, but they are mainly applicable to the earlier amiloride-induced fluctuation analyses. Since we were primarily interested in determining changes to the total number of channels, we elected to perform our studies with fluctuation analysis. Keeping in mind some of the objections mentioned above, we decided to make use of two new methods of 6-chloro-3,5-diamino-pyrazine-2-carboxamide (CDPC)-induced fluctuation analysis (see Methods). CDPC is a weak electroneutral  $\text{Na}^+$  channel blocker which allowed us to perform our measurements at conditions very close to the physiological state of the tissues. We also used a specially designed chamber for the experiments and utilised a state-of-the-art technology, low-noise voltage clamp, especially designed for fluctuation analysis (Dr. W. Van Driessche, University of Leuven, Belgium), in order to reduce the  $1/f$  noise to a minimum. The tissues were studied under short-circuit conditions which provided valuable information about the rates of  $\text{Na}^+$  transport. The currents are easily measured with very high precision so that even minor changes of current may be analysed to obtain information about the mechanism of action of aldosterone (Helman and Baxendale, 1990). Under these conditions, the values of channel corner frequency and rate coefficients and, hence, values of  $i_{\text{Na}}$ ,  $P_o$ , were remarkably similar to those obtained for  $\text{Na}^+$  channels with patch clamp (Palmer and Frindt 1986a). The

steady-state experiments supplied us with information on changes to the channels caused by aldosterone after periods of 24, 48 and 72 hours. We were also curious to know how the  $\text{Na}^+$  channels would respond to aldosterone during periods of many hours and when the transport responses are complex, as happens during the early phase of the response to aldosterone. Towards this, we made use of a new and rapid method to measure specifically the  $P_o$  and channel densities at discrete short intervals (Helman *et al.*, 1995). The advantage gained by this method is that the tissues can be studied at discrete times to resolve aldosterone-induced changes to the channel parameters far removed from control. In this way we could examine the changes during the early and late phases of aldosterone action. We have already presented preliminary results stemming from this study (Baldwin and Els, 1995).

Since many, especially the recent immunocytochemical and electrical studies are performed on cultured A6 cells, we chose to perform our experiments on similar cells cultured under standard conditions. While not using native tissues, we could compare our results directly with others on A6 cells minimising the additional problem of species differences.

We performed this thesis with the idea that we may find additional and new evidence to discriminate between the theories of the action of aldosterone towards a unified concept for its important mechanisms of action.

## Chapter 2

# Materials And Methods

---

---

## 2.1 Cell Culture Methods

A6 cells (Johnson clone) at passage 106 (a gift from Professor W. van Driessche of the K.U. Leuven, Belgium) were stored in cryovials filled with freezing medium in liquid nitrogen. From these, cells were defrosted and grown up in 260 ml culture flasks (Nunclon<sup>®</sup> Delta) to obtain a seed stock. The culture medium was a Delbecco's Modified Eagle Medium (DMEM) (Highveld Biological) to which 1,041 g/l of Hepes (sodium salt, Fw: 260.3 g/mol) (Sigma) was added as a buffer. The final pH was adjusted to 7.4. Medium was filtered using a 2 µm hollow fibre media filter (MediaKap<sup>™</sup>-5 or Millipore) and a peristaltic pump (Masterflex<sup>®</sup> pump controller Cole Parmer Instrument Co.) into 500 ml autoclaved bottles. The DMEM was supplemented with 50 ml of heat inactivated (30 minutes in a 56 °C waterbath) fetal calf serum (FCS) (Highveld Biological), added to every 450 ml filtered medium. To combat infection, 5 ml of an antibiotic solution containing penicillin (100 µg/ml) and streptomycin (100 units/ml) was added to each 500 ml medium plus FCS. The final osmolality of the medium was approximately 250 mOsm/kg. Flasks were placed in a 28 °C, humidified incubator with 5% CO<sub>2</sub> at a pressure of 3 kPa. The medium was changed initially after one day and subsequently after every two days.

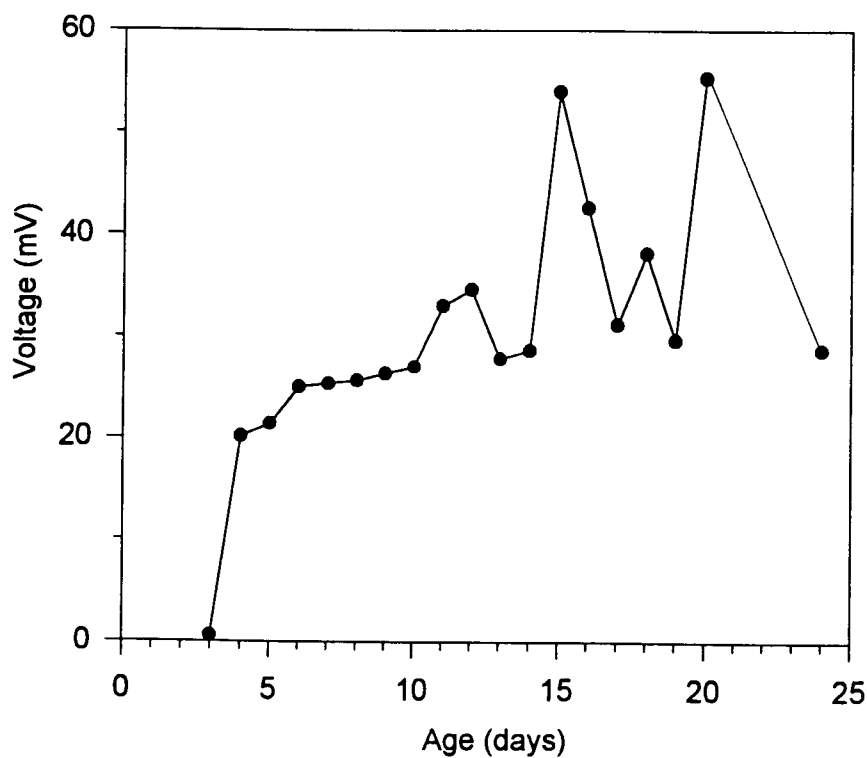
Cells maintained on plastic culture flasks were split on confluency (about 5 days) and either subcultured or frozen down (see appendix). The cells were lifted once weekly by washing with 15 ml phosphate-buffered saline (PBS, composition in mM: 8.5 NaCl; 17.5 NaHCO<sub>3</sub>; 4 KCl; 0.8 K<sub>2</sub>HPO<sub>4</sub>) followed by 5 ml of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS at pH 7.4. The cells were dissociated from the flasks within 10 minutes at 28 °C after which 5 ml medium was added to the flasks to inactivate the trypsin. The

contents of the flasks were then removed and placed into 10 ml centrifuge tubes. Cells were spun down at 1000 g for 2 minutes. The supernatant was discarded and the pellet of cells re-suspended in 10 ml culture medium. Cells grown on non-porous surfaces, such as plastic, do not form polarized epithelial monolayers (Lewis and Donaldson, 1990). There are a number of artificial membranes available on which cells can be grown to allow them to form differentiated epithelial monolayers. To be consistent with the growth of A6 epithelia in many laboratories, it was decided to use Millicell™-HA culture plate inserts (Millipore), which comprise of a perspex cup (30 mm in diameter) and a nitrocellulose membrane (pore size: 0.45  $\mu\text{m}$ ) on the bottom. 6 inserts were placed into wells of Nunc, 6-well multidishes. To bathe the basolateral surface of the inserts, 2 ml medium was placed into each of the wells before the inserts were lowered into this medium to allow wetting. Cells were counted with the aid of a Nebula counting chamber to determine the re-suspension density and the appropriate quantity of re-suspended cells in medium was placed in the cup of the inserts such that the seeding density was about  $1 \times 10^5$  cells/cm<sup>2</sup> when the total amount of medium on the apical side was brought up to 3 ml. Cells on their inserts, in 6-well multidishes, were incubated until required for the electrophysiological experiments. Medium of the inserts was replaced initially after 2 hours and then every second day to remove dead cells and to replenish nutrients and growth factors.

### ***2.1.1 Selection of Inserts for Electrophysiological Experiments***

Transepithelial voltages ( $V_T$ ) of cells growing on inserts were measured, in their wells, with the aid of a Millicell®-Electrical Resistance System (Millipore). These voltages gave an indication of whether the cells were transporting ions (according to Ohm's law,  $V$  is proportional to  $I$ ) and an indication of whether the cells had formed a confluent monolayer. As seen in Fig. 2.1, significant  $V_T$ 's were measured after about 5 days which coincides with the observation using an inverted microscope, that A6 cells grown on Nunc filters, reached confluency around this age. Thereafter, voltages steadily increased until around 10 days,

from when the voltages remained consistently high (averaging above 25 mV). It appears that after this time, the age of the inserts does not have great effects on the transport properties of the cells. Accordingly, cell monolayers between 10 and 28 days old and exhibiting  $V_T$ 's that were greater than 20 mV were selected for electrophysiological experiments. Confluent monolayers of cells on their inserts were carefully mounted into the chamber as described below. Open circuit  $V_T$ 's were again measured in the chamber and any inserts that showed a decrease in their  $V_T$  of  $> 50\%$  were discarded since the monolayer had possibly been disturbed. Cells from passages 107 to 114 were used in the experiments.



**Fig. 2.1** Age distribution of transepithelial voltages of A6 cells

*This graph shows the average  $V_T$ 's recorded from a pool of 106 inserts, in their wells, at various days after seeding. This mainly illustrates the trend of  $V_T$ 's to increase over time and the reason experiments were performed on cells older than 10 days.*

## 2.2 Experimental Procedures

We employed two different types of electrophysiological experiments on cultured A6 cells:

1) paired steady state experiments and 2) time course experiments.

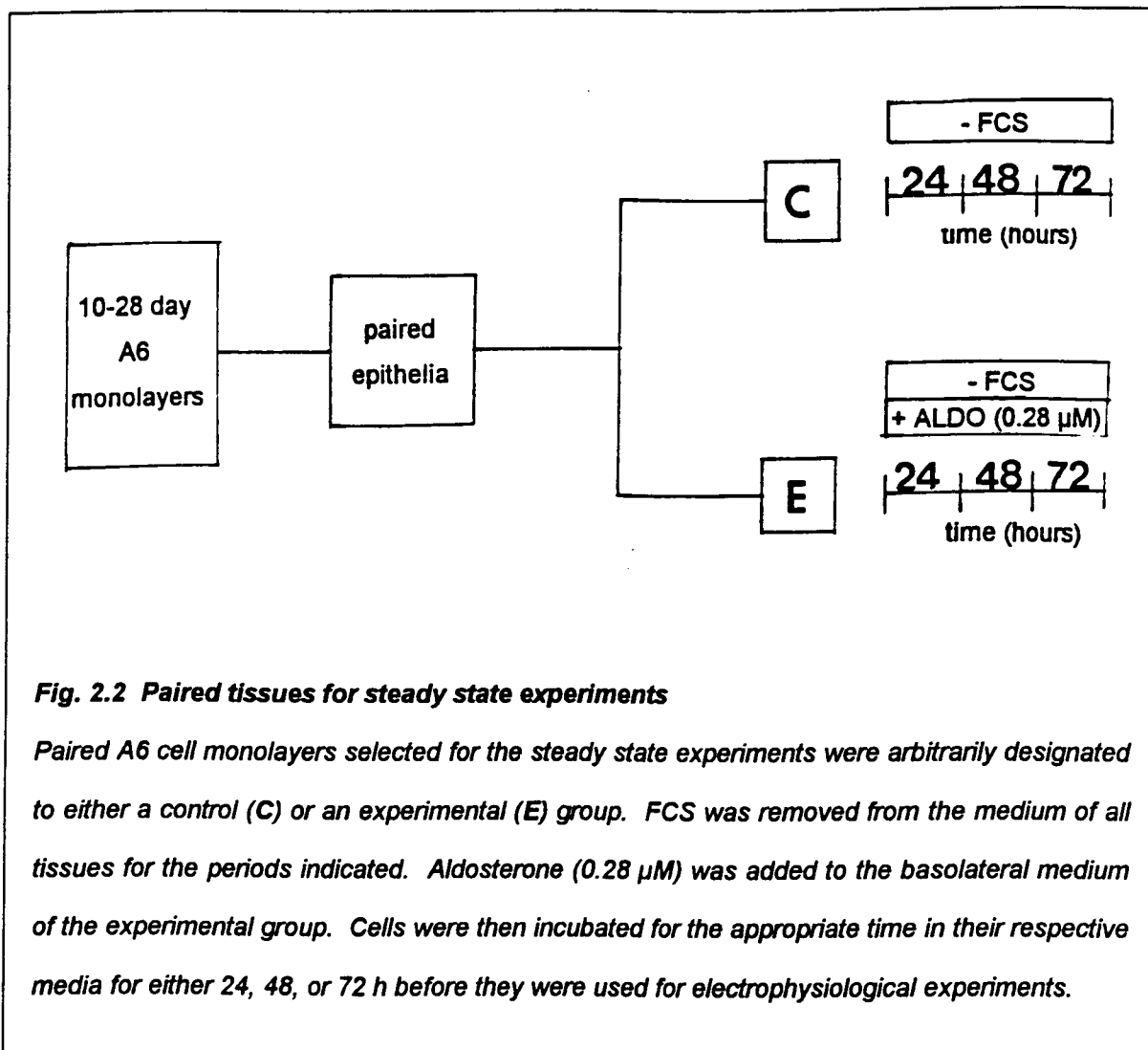
### 2.2.1 Tissue Treatment

#### 2.2.1.1 Paired Tissues for Steady State Experiments

The protocol outlined below for the steady state experiments allowed us to examine the effects of prolonged aldosterone exposure on cultured epithelia. For each steady state experiment, two inserts that had been growing under identical conditions and displaying similar  $V_T$ 's ( $> 20$  mV) were selected (hereafter referred to as paired tissues) and were randomly assigned to either the control group or the experimental group. Since FCS may contain trace amounts of mineralocorticoids, it was omitted from the culture medium of paired inserts at least 24 hours before each experiment. During this time the effects of mineralocorticoids should have abated significantly, seeing as the half-life of aldosterone is about 7 hours (Garty, 1986). To cells destined to become the experimental tissue, d-aldosterone (Sigma) at a final concentration<sup>2</sup> of 0.28  $\mu$ M was added to the basolateral medium. The other tissue remained untreated and formed the control. Both inserts were then incubated for periods of either 24, 48 or 72 hours before being removed from the incubator for the electrophysiological experiments (Fig. 2.2). Hence control and experimental monolayers were always the same age, same passage, from the same 6-well tray and were treated identically except for the addition of the hormone. Noise analyses were performed on both these inserts on the same day (see details below).

---

<sup>2</sup>We decided on a final aldosterone concentration of 0.28  $\mu$ M as this falls in the physiological range of the hormone (Kizer *et al.*, 1995) and it is the concentration used by our collaborators (Helman and colleagues).



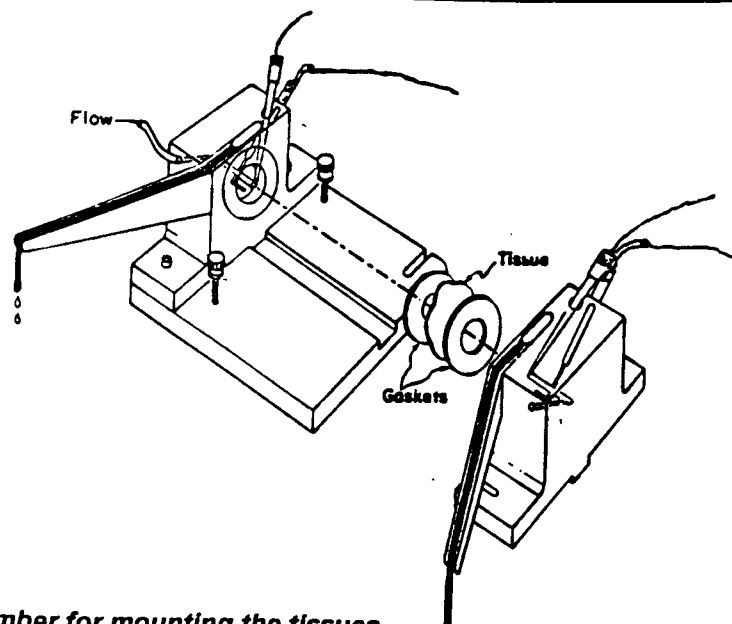
### 2.2.1.2 Tissues for Time Course Experiments

For each time course experiment, one insert ( $V_T > 20$  mV) was selected and the FCS was omitted from the growth medium 24 hours before the experiment. Electrophysiological experiments, following a pulse inhibition protocol (described below), were performed on a single insert. After a stabilisation period of around 3 hours, when the  $I_{SC}$  reached apparent steady state levels, noise analyses were performed on the tissue over a period of about 2 hours to determine control parameters for  $\text{Na}^+$  transport. 0.28  $\mu\text{M}$  aldosterone was added to the medium perfusing the basolateral surface of the epithelium (taken as time zero) and noise analyses were performed at regular intervals of 20 min for up to 13 hours.

## 2.2.2 Electrophysiological Procedures

### 2.2.2.1 General setup

Inserts for electrophysiological experiments were taken from the incubator and excess fluid was removed from the lower surface of each insert by touching a tissue to its bottom edge. Each insert was then glued onto a polycarbon (perspex) transfer ring, approximately 15 mm in diameter, using Super Glue - a cyanoacrylate tissue glue (Eurecyl™, Silicone Technical Products, South Africa). A sharpened metal tissue punch with a diameter slightly larger than the transfer ring was used to cut out the insert membrane. The ring with its attached tissue was transferred to a modified Ussing chamber (Fig. 2.3) where it was held in place by two gaskets with a smear of silicone grease (Dow Corning high vacuum grease) to minimise edge damage (Helman and Miller, 1971) and to obtain a tight seal. Tissues were bathed appropriately with "control and "experimental" medium.

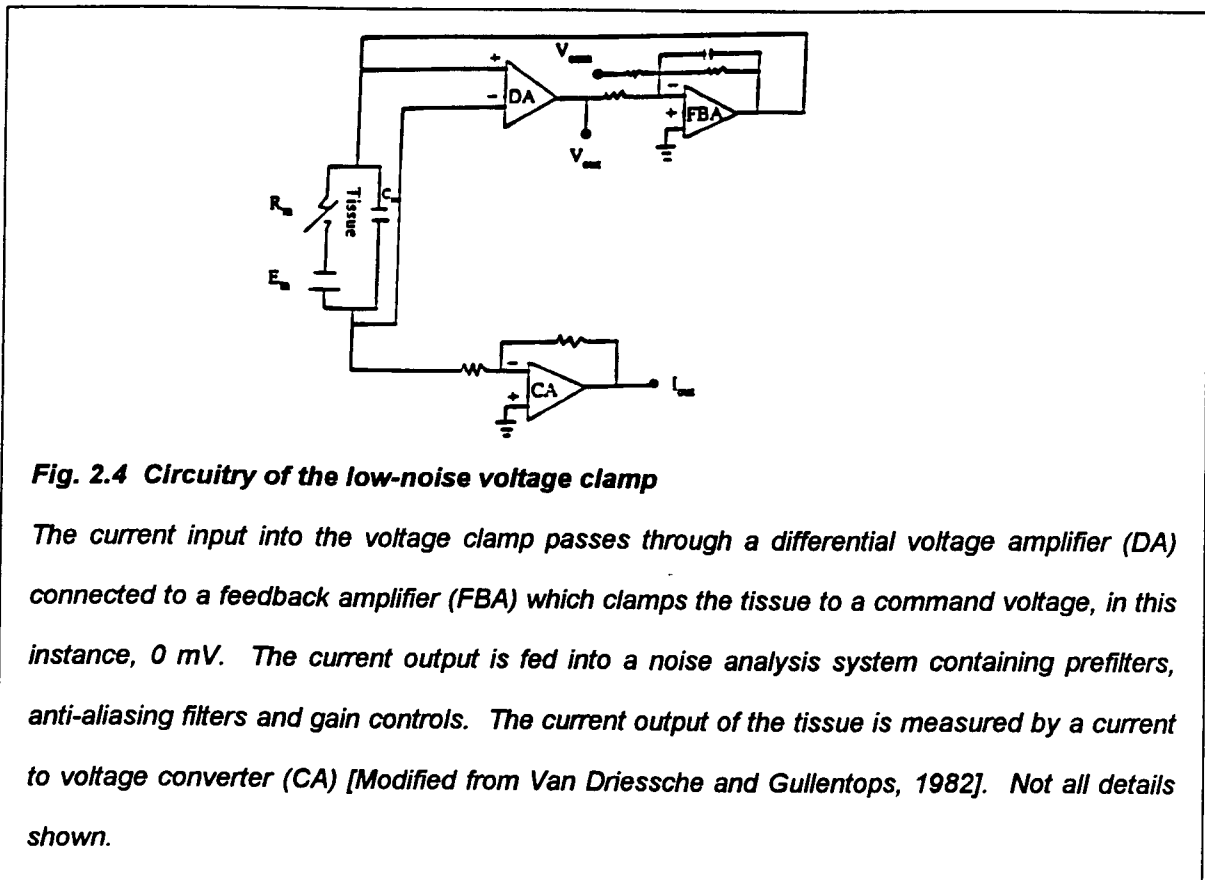


**Fig. 2.3 Chamber for mounting the tissues**

*This diagram illustrates the modified Ussing chamber used to permit continuous flow (9 ml/min) of solutions during noise analysis. Fluid enters the chambers by gravity via polyethylene tubing and spillways containing wicks made from paper tissue allow the solution to leave the chamber. The volume of each half of the chamber is approximately 0.6 ml and the area of tissue exposed to the bathing solutions is 0.75 cm<sup>2</sup>. Low resistance bridges for paired current and voltage electrodes are connected via Ag/AgCl wires to the voltage clamp [Abramcheck et al., 1985].*

### 2.2.2.2 Set-up for Noise Analysis

Tissues were mounted in a modified Ussing chamber and connected to the noise analysis equipment with a four electrode system (see appendix). The chamber, with the tissue mounted and electrodes in place, was set up in a shielded (Faraday) cage supported on a concrete block to minimise vibrations. Electrical connections were earthed and the voltage clamp apparatus was run off a DC battery ( $\pm 15$  V) to reduce the possibility of AC interference from the equipment. Experiments were performed at room temperature, although on colder days a convection heater was used to maintain the room temperature as close to 20 °C as possible. The macroscopic current was continuously recorded by a chart recorder (Linear 1200, Anatech Instruments Ltd., South Africa). The tissues were continuously short-circuited with a low-noise voltage clamp (Fig. 2.4) (Van Driessche and Lindemann, 1978; Van Driessche and Gullentops, 1982). Under short-circuited conditions there is minimal movement of ions via the paracellular pathway such that the  $I_{sc}$  represents almost exclusively the ion passage across the transcellular pathway.



**Fig. 2.4** Circuitry of the low-noise voltage clamp

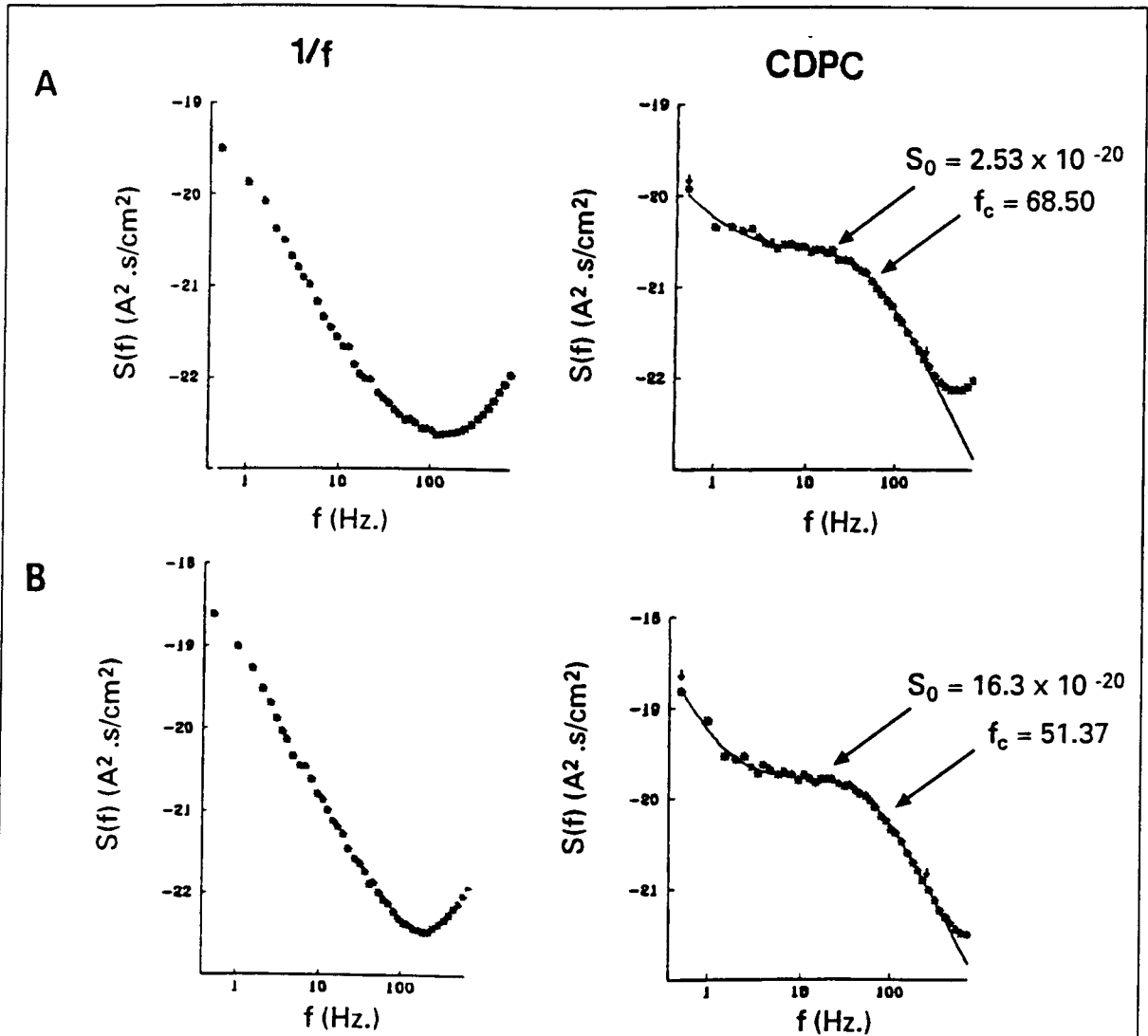
The current input into the voltage clamp passes through a differential voltage amplifier (DA) connected to a feedback amplifier (FBA) which clamps the tissue to a command voltage, in this instance, 0 mV. The current output is fed into a noise analysis system containing prefilters, anti-aliasing filters and gain controls. The current output of the tissue is measured by a current to voltage converter (CA) [Modified from Van Driessche and Gullentops, 1982]. Not all details shown.

### 2.2.2.3 Data Capturing and Processing

The set up provided a means of continuously recording the transepithelial conductance (see later) and the  $I_{sc}$  across the cell monolayer. In order to analyse the current fluctuations during noise analysis, the AC and DC components of the  $I_{sc}$  were separated by a high-pass rectifying current filter and the AC current was amplified 1000 times. After the high amplitude AC component was filtered out by an anti-aliasing low-pass filter, the signal was digitised with an analogue-to-digital (A/D) converter (RTI 800, Analog Devices Inc., Norwood, Mass., USA.) (as described by Desmedt *et al.*, 1993). The signal was sampled at a fundamental frequency of 0.5 Hz. which produced 4096 points collected over a 2 s period. These points were converted from the time domain to frequency domain by means of a fast Fourier transform software program (algorithm described by Cooley *et al.* 1967) to derive power density spectra (PDS). Averaged PDS of 2048 frequencies were obtained from 50 sweeps of data collection at each concentration of blocker. The digitised signals were monitored on an oscilloscope and recorded on an IBM<sup>®</sup> compatible 486 computer using a data acquisition program "Noise" obtained from Professor W. van Driessche, K.U. Leuven and later stored on disc (De Wolf and Van Driessche, 1986). This software was programmed in accordance with standard analysis procedures by K. Wessels, and the hardware was developed by G. Raskin (K. U. Leuven). The corner frequency ( $f_c$ ) and the plateau value ( $S_o$ ) are two important parameters that are gained from the PDS and these form the corner stones of our calculations that reveal the channel parameters. Values of  $S_o$  and  $f_c$  were determined by non-linear least squares regression analysis (Brown and Dennis, 1972) from the PDS using a customised software program. For noise following exponential kinetics, the PDS of the fluctuations in current consists of low frequency noise and a single Lorentzian function [S(f)] according to the following scheme:

$$S(f) = \frac{S_o}{1 + (f/f_c)^2} + \frac{A}{f^\alpha} \quad (\text{eq. 1})$$

where  $A$  is the amplitude of the low-frequency noise component at  $f = 1$  Hz. and  $\alpha$  is its slope. Hence the values of the corner frequency,  $f_c$  and  $S_0$  were determined from the curve (Fig. 2.5)



**Fig. 2.5 Power density spectra (PDS)**

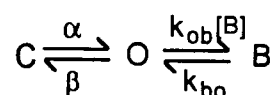
The PDS show  $1/f$  noise (at  $0 \mu M$  CDPC) and Lorentzians recorded at  $30 \mu M$  CDPC from paired control tissue (A) and tissue that was exposed to aldosterone (B) for 24 hours. CDPC induced current fluctuations produced a single Lorentzian in the PDS. The Lorentzians dominate in the mid frequency range (10-400 Hz) of the spectra, whereas the inherent noise of the membrane ( $1/f$  noise) is prevalent at very low frequencies. Amplifier noise dominates at the higher frequency end of the spectra. The values of  $f_c$  and  $S_0$  are determined from the curve and used for calculations that resolve the channel parameters.

To aid analyses, data was entered into a spreadsheet in the Lotus 1,2,3 program. Equations for current fluctuation analysis were programmed into the spreadsheet with automatic calculation on entry of data. Hence the macroscopic and molecular parameters affecting the membrane permeability were determined. Statistical data are presented as means  $\pm$  the standard error of the mean (SEM). On paired data, the Student's *t* test was carried out, using a statistical package, Sigmaplot, to determine the significance (at  $P < 0.05$ ) of differences between means.

#### 3.2.2.4 Blocker Induced Fluctuation Analysis

$\text{Na}^+$  channels spontaneously fluctuate between open and closed states. While the  $\text{Na}^+$  channels of excitable membranes undergo these fluctuations at a fast rate (msec), those in epithelial membranes fluctuate relatively slowly (in the seconds range) (Helman and Van Driessche, 1990). The spontaneous opening and closing of epithelial membrane  $\text{Na}^+$  channels border on the limits of the resolution of the equipment, being too slow to analyse with accuracy. Van Driessche and Lindeman (1978) developed a method whereby the addition of a channel blocker is used to induce fluctuations between an open and a blocked state of the channel at a faster rate than the spontaneous open and closed fluctuations. These blocker-induced fluctuations are measurable by the equipment.

The weak electroneutral  $\text{Na}^+$  channel blocker 6-chloro-3,5-diamino-pyrazine-2-carboxamide (CDPC) was used to produce blocker-induced current fluctuations. CDPC current fluctuations are brought about as a result of the interaction of the blocker with  $\text{Na}^+$  channels in the open state. We assumed a simple three state model to describe the distribution of channels between closed, open and blocked states. The association and dissociation of blocker molecules with open channels are given by the rate coefficients  $k_{ob}$  and  $k_{bo}$  respectively. The rate coefficients  $\alpha$  and  $\beta$  describe fluctuations of open to closed and closed to open states respectively:



### Staircase Protocol for Steady State Experiments

Experiments following the staircase protocol, described by Helman and Baxendale (1990), enabled the determination of Na<sup>+</sup> channel parameters under steady state conditions. Tissues were set-up as described, short circuited and allowed to stabilise until apparent steady state conditions (indicated by a constant  $I_{sc}$ ) were achieved (see Fig. 3.2, Results). The transepithelial membrane conductance ( $G_T$ ) could be measured by imposing a brief 3 mV automated, unipolar voltage pulse across the membrane, every 15 s. The resultant change in  $I_{sc}$ , also recorded with a multimeter, caused an upward deflection in the trace (see Fig. 3.2, Results). Hence the membrane conductance could be calculated using Ohm's Law and where  $G = 1/R$ . The height of the deflection is an indication of the magnitude of  $G_T$ . A higher  $G_T$  being indicated by a thicker line (larger deflection). The pulse was turned off during periods of noise analyses. Noise analysis involved the stepwise addition of increasing concentrations of CDPC (5 to 50  $\mu$ M) to the apical solution and measuring the resultant microscopic current fluctuations with a low noise amplifier.

Data for the PDS were recorded after the maximal inhibition at each blocker concentration. There was a small amount of autoregulation of Na<sup>+</sup> channels noticeable a) by the positive slope of the trace following peak inhibition at each [CDPC] and b) at the end of noise analysis, when the CDPC was washed out and replaced with the original medium (without blocker), the  $I_{sc}$  recovered to a level higher than it was before the introduction of blocker (Fig. 3.2, Results). However, the  $I_{sc}$  returned to steady state values within about 20 minutes, after the blocker was washed out. By adding 100  $\mu$ M amiloride to the apical solution at the end of each experiment, the portion of the  $I_{sc}$  carried by Na<sup>+</sup> ions, the  $I_{Na}$ , could be determined from:

$$I_{Na} = I_{sc} - I_{amiloride} \quad (\text{eq. 2})$$

where the amiloride insensitive current,  $I_{\text{amiloride}}$ , is the portion of the  $I_{\text{SC}}$  carried by ions other than  $\text{Na}^+$ .

The rate coefficients of the blocker interactions with  $\text{Na}^+$  channels were calculated from a plot of  $2\pi f_c$  vs. blocker concentration, [B]. This produced a straight line indicating a first order reaction, and hence supporting the choice of the three state model to describe the blocker reaction. The rate coefficients  $k_{\text{ob}}$  and  $k_{\text{bo}}$  were determined respectively, from the slope and the y-intercept of the rate-concentration plots according to the equation:

$$2\pi f_c = k_{\text{ob}}[\text{B}] + k_{\text{bo}} \quad (\text{eq. 3})$$

The equilibrium blocker coefficient of open channels was calculated from :

$$K_B = \frac{k_{\text{ob}}}{k_{\text{bo}}}$$

Accordingly, the mean  $i_{\text{Na}}$  at each [B] could be calculated using the values for  $I_{\text{Na}}$ ,  $S_o$ ,  $f_c$  and  $k_{\text{ob}}$  according to the equation:

$$i_{\text{Na}}^{\text{B}} = \frac{S_o \times (2\pi f_c)^2}{4 \times I_{\text{Na}} \times k_{\text{ob}} \times [\text{B}]} \quad (\text{eq. 4})$$

Note that the superscript (B) included with the usual notations indicates that a measurement was made in the presence of blocker. Extrapolation to zero [B] provides an estimation of values in the absence of blocker (see Fig. 3.1D, Results).

Open channel density ( $N_o$ ), in the absence of blocker, was calculated from:

$$N_o = \frac{I_{\text{Na}}}{i_{\text{Na}}} \quad (\text{eq. 5})$$

To determine the channel open probability, we took advantage of the fact that CDPC interacts only with open channels. The defining relationship of the three states of the channel is given by the following equation (Helman and Baxendale, 1990)

$$\frac{N_o^B}{N_o} = \frac{1}{1 + P_o (B/K_B)}$$

and

$$P_o = \frac{1 - N_o^B/N_o}{(N_o^B/N_o) ([B]/K_B)} \quad (\text{eq. 6})$$

Hence the open probability and the blocker equilibrium constant uniquely define the density of open channels at any blocker concentration, relative to the density of open channels in the absence of blocker.

The sum of electrically active open and closed channels in the absence of blocker, equivalent to the total channel density ( $N_T$ ), was calculated from:

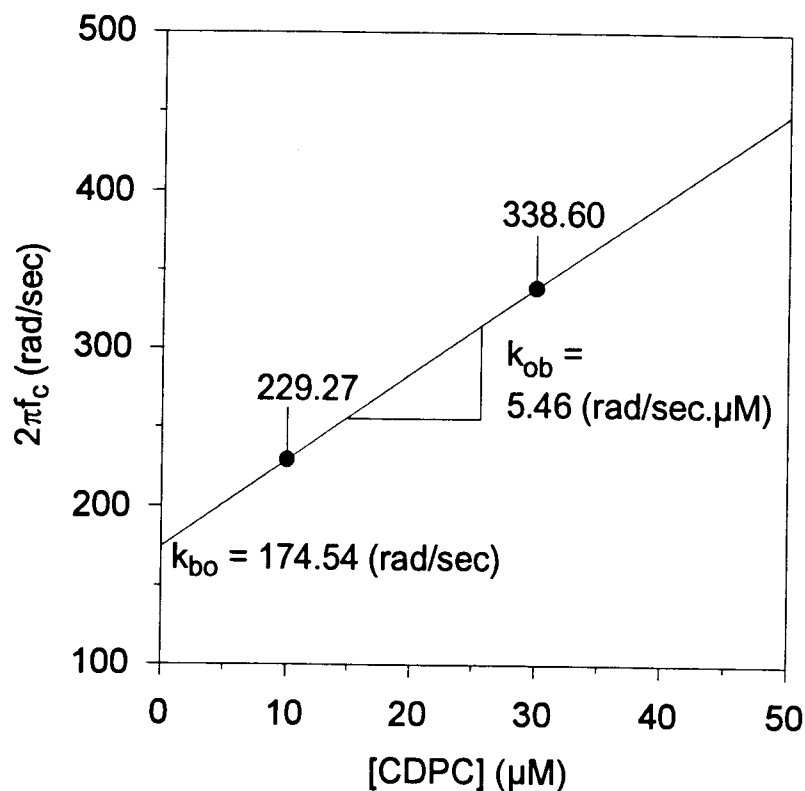
$$N_T = (N_o + N_c) = \frac{N_o}{P_o} \quad (\text{eq. 7})$$

### Pulse Inhibition Protocol for Time Course Experiments

The staircase protocol of blocker-induced noise analysis has been limited to experimental conditions where the tissues are in apparent steady states of transport. To examine the early effects of aldosterone, it was necessary to use a method that would permit determination of the single channel parameters over many hours and also during transient periods of  $\text{Na}^+$  transport. In this regard, we employed a new method of noise analysis referred to as the pulse inhibition method (Helman *et al.* 1995)

Briefly, this method depends on only two measurements, namely the current-noise PDS's and the inhibition of the  $I_{sc}$  by the  $\text{Na}^+$  channel blocker, CDPC, added to the apical medium. During an experiment, tissues are setup in exactly the same manner as already described for the stepwise protocol and the  $I_{sc}$  is allowed to stabilise. Thereafter tissues are continuously exposed to a low concentration (10  $\mu\text{M}$ ) of CDPC and after another stabilising period, the blocker induced Lorentzians can be measured at any time. At selected periods,

the tissues are exposed to a slightly higher concentration (30  $\mu\text{M}$ ) CDPC solution causing relatively small inhibitions of the  $I_{\text{SC}}$  averaging about 8 % (see Fig 3.4, Results). New blocker-induced Lorentzians are measured when the  $I_{\text{SC}}$  reaches its minimal value under the influence of 30  $\mu\text{M}$  CDPC (i.e. maximal inhibition by the channel blocker). From these two sets of values, the rate concentration plots can be produced. Since  $f_c$  increases linearly with blocker concentration, the blocker on- and off-rate coefficients can be determined from the slope and y-intercept of the rate concentration plots (Fig. 2.6). Once these and the apparent equilibrium constant,  $K_B$ , are known,  $i_{\text{Na}}$  and  $N_O$  are calculated in the usual manner described above.



**Fig. 2.6 Rate concentration plot**

*Illustrated here is an example of a rate concentration plot which is a linear regression on the two  $2\pi f_c$  data points determined at 10 and 30  $\mu\text{M}$  CDPC by the pulse inhibition protocol method of noise analysis. The blocker on- and off- rate coefficients are obtained from the values of the slope and y-intercept respectively. These values are used to calculate the single channel current and open channel density.*

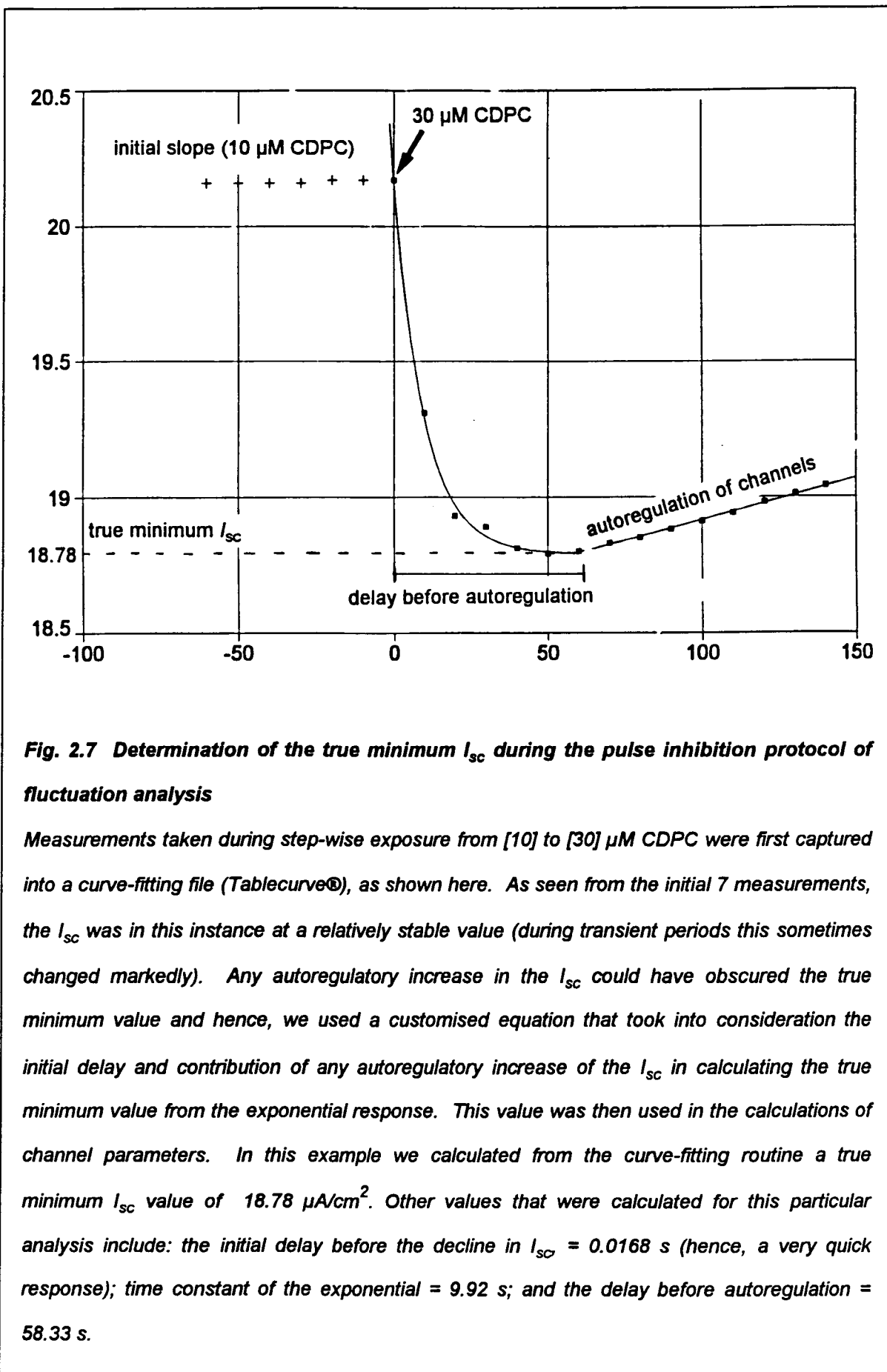
Since the ratios of open channel densities (determined from the ratios of macroscopic currents and single channel currents at 10 and 30  $\mu\text{M}$  CDPC) are already known, channel  $P_o$  could be calculated directly according to the following equations :

$$\frac{N_o^{30}}{N_o^{10}} = \frac{1 + P_o(10/K_B)}{1 + P_o(30/K_B)} = \left[ \frac{I_{Na}^{30}}{I_{Na}^{10}} \right] \left[ \frac{i_{Na}^{10}}{i_{Na}^{30}} \right]$$

and

$$P_o = \left[ \frac{1 - (I_{Na}^{30}/I_{Na}^{10})}{30(I_{Na}^{30}/I_{Na}^{10}) - 10} \right] K_B$$

When measuring  $P_o$ , it is necessary to take cognisance of the time taken for the channels to redistribute themselves amongst the closed, open and blocked states. This, and other time-related factors, need to be considered when determining the kinetics of blocker inhibition of the macroscopic currents (Helman *et al.*, 1995). There are two relatively fast time constants associated solely with blocker inhibition of the channels. The minimum time required to achieve steady states is about 6 to 8 seconds following the start of blocker inhibition (Dr. Helman, personal communication). Next is the time necessary for the exchange and mixing of the solutions when switching from 10 to 30  $\mu\text{M}$  CDPC due to the unstirred layers at the surfaces of the cells and the dimensions of the chamber and the input lines. Taking these into account, steady state redistribution of the channels should be completed within about 40 to 60 seconds. In addition, autoregulatory increases of channel density occur, usually after a delay of about 30 to 60 seconds. Hence, to determine changes of  $I_{SC}$  associated with inhibition of open channels, we used a non-linear curve fitting routine to fit the data to an equation that took into account baseline drift, mixing time and the delayed autoregulatory increases in channel density (Fig. 2.7).



Values of the current were recorded at intervals of 10 seconds for about a minute prior to increasing the concentration of CDPC and thereafter for about 3 minutes (Fig 3.5). It became clear that the values of the channel parameters calculated during control periods with the two methods of current noise analysis were remarkably similar. Importantly, the critical time of measurement with the pulse protocol is about 60 seconds or less, allowing rapid determination of changes to the Na<sup>+</sup> channels, even during transient changes to Na<sup>+</sup> transport and hence perturbation of the system is minimized.

## Chapter 3

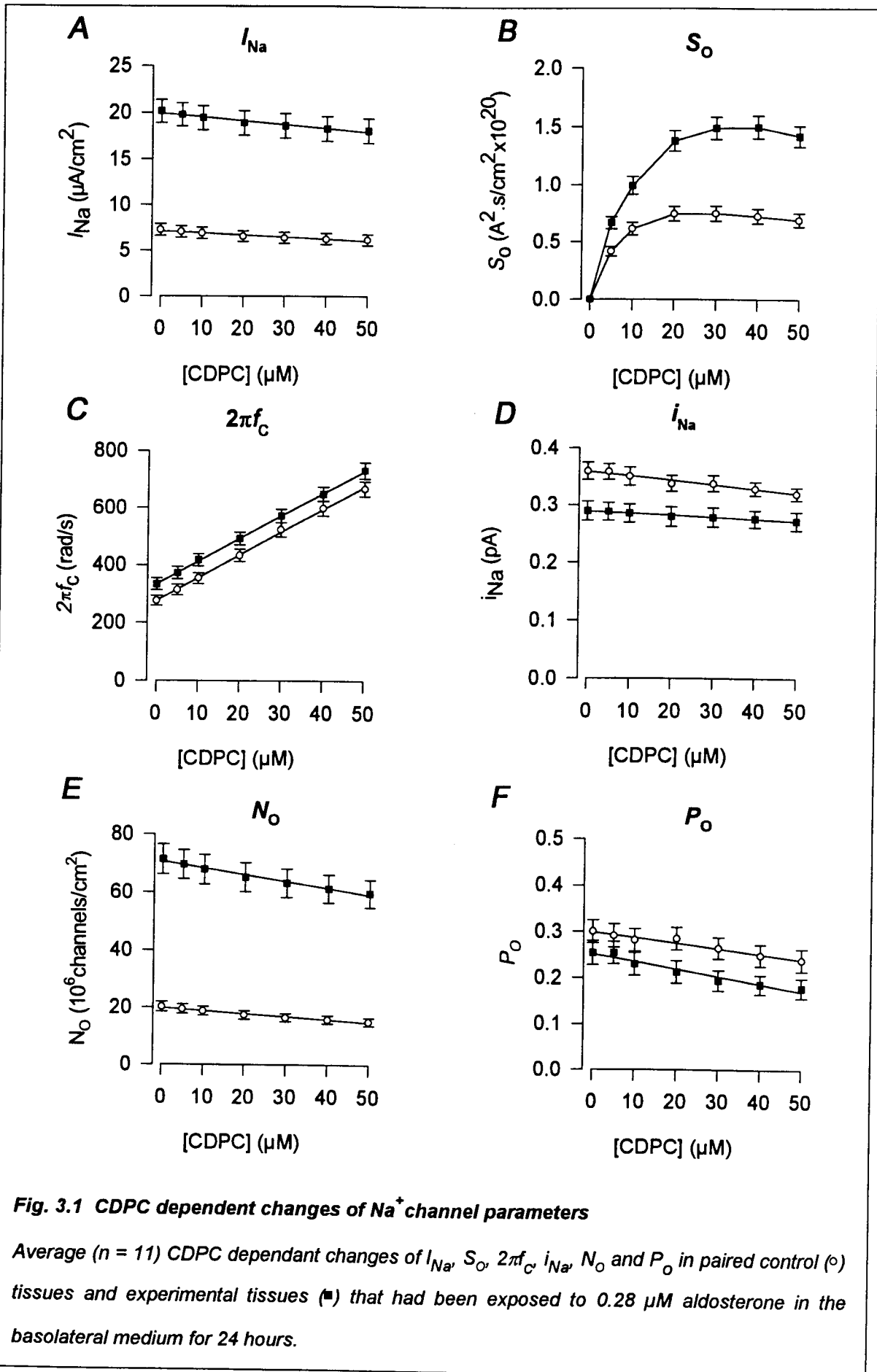
# Results

---

### 3.1 Control baseline values

It was important to first resolve baseline values for the transport properties of the A6 cells used in our experiments (Table 1). This was especially important to enable comparisons with baseline values and properties of the  $\text{Na}^+$  channels in A6 cells reported by other laboratories using similar and different methods. A representative summary of the data used in our calculations appear in Fig. 3.1 and the values are summarised in Table 2. As can be seen, the introduction of CDPC for noise analyses did not cause large perturbances in the macroscopic transport rates of the tissues. The mean variation of  $I_{\text{Na}}$ 's with increasing blocker concentrations are shown in Fig. 3.1A. CDPC produced small graded inhibitions of the  $I_{\text{Na}}$  with increasing blocker concentration from 0 to 50  $\mu\text{M}$ , the maximum inhibition being less than 20 %. Moreover, the time taken to perform noise analysis was less than 12 minutes. Thus, importantly, the tissues remained close to their physiological rates of  $\text{Na}^+$  transport during noise analysis (also see Fig. 3.2).

$S_0$  values were obtained directly from the PDS (see Materials and Methods). As predicted from equation 1, CDPC brought about the expected biphasic relationship in the response of  $S_0$  to increasing blocker concentration (Fig. 3.1B). The  $f_c$  values, measured from the PDS, increased linearly with increasing CDPC concentrations (Fig. 3.1C;  $r^2 = 0.99$  by linear regression), consistent with a three state model to describe the  $\text{Na}^+$  channel kinetics. The rate coefficients were calculated from a plot of  $2\pi f_c$  vs. [CDPC] (eq. 3). The value of  $k_{\text{ob}}$  was obtained from the slope of the graph and the y-intercept provided the value for  $k_{\text{bo}}$ . The apparent  $K_B$  was calculated from  $\frac{k_{\text{ob}}}{k_{\text{bo}}}$ .



Thereafter the values obtained for  $I_{Na}$ ,  $k_{ob}$ ,  $f_c$  and  $S_o$  were used to calculate the  $i_{Na}$  at each [CDPC] according to equation 4 (see Materials and Methods). Surprisingly, increasing blocker concentrations brought about decreases in  $i_{Na}$  (Fig. 3.1D). It was expected that the small inhibitions of  $I_{sc}$  with a step-wise increase in [CDPC] would cause parallel increases in  $i_{Na}$  due to small increases of the fractional transcellular resistance and hyperpolarization of apical membrane voltage (Helman and Baxendale, 1990). In our experiments, this did not happen and rather, as seen in Fig. 3.1D,  $i_{Na}$  decreased with increasing [CDPC]. Such phenomena may be expected especially at relatively low transport rates, as found in A6 cells, and when the fractional transcellular resistances are low and also where autoregulatory increases of blocker-sensitive currents are significant, as seen in Fig. 3.2 (Els and Helman, 1991). If autoregulation also involves an increase in blocker-insensitive currents, then with increasing [CDPC] and time inhibition, the  $i_{Na}^B$  would appear to decrease due to an increase in  $i_{Na}^B$  that would not be reflected in the values of  $S_o$  that report only noise from blocker-sensitive channels (Els and Helman, 1996). Regardless, and since the decreases in  $i_{Na}$  were small (Fig. 3.1D), extrapolation of the  $i_{Na}^B$  to zero [CDPC] by linear regression provided values of  $i_{Na}$  that were independent of any autoregulatory increases in channel density.

Accordingly, the value of  $N_o$  at each [CDPC] was calculated from the quotient  $I_{Na}/i_{Na}$  and  $N_o$  in the absence of blocker was determined by linear regression (Fig. 3.1E).  $P_o$  was calculated at each [CDPC] from equation (eq. 6) and  $P_o$  decreased with increasing [CDPC] and its value in the absence of blocker was determined by linear regression (Fig. 3.1F).

**Table 1: Summary of baseline control parameters of cultured A6 cell epithelia**

$I_{Na}$ ( $\mu\text{A}/\text{cm}^2$ )	$i_{Na}$ (pA)	$P_o$	$N_o$ ( $10^6/\text{cm}^2$ )	$N_T$ ( $10^6/\text{cm}^2$ )	$k_{ob}$ (rad/s. $\mu\text{M}$ )	$k_{bo}$ (rad/sec)	$K_B$ ( $\mu\text{M}$ )
7.20	0.31	0.30	24.13	106.81	7.63	232.55	30.38
$\pm 0.89$	$\pm 0.01$	$\pm 0.02$	$\pm 3.67$	$\pm 23.95$	$\pm 0.21$	$\pm 13.82$	$\pm 1.46$

Values expressed as means  $\pm$  S.E.M. (n = 27)

Presented in Table 1 is a summary of the baseline values for the parameters of apical membrane  $\text{Na}^+$  channels as determined during control periods of all our experiments. Data were recorded from 27 non-aldosterone stimulated inserts between 10 and 28 days after seeding. The mean  $V_T$  was  $30 \pm 3$  mV, the  $R_T$  averaged  $2.3 \pm 1$   $\text{k}\Omega.\text{cm}^2$  and the mean  $I_{SC}$  was  $8.3 \pm 1.3$   $\mu\text{A}/\text{cm}^2$ . The individual values of  $I_{SC}$  varied very widely (range: 1.83 to 36.25  $\mu\text{A}/\text{cm}^2$ ) among inserts, while the  $I_{Na}$ , representative of the total macroscopic  $\text{Na}^+$  transport, averaged 7.2  $\mu\text{A}/\text{cm}^2$ . These values were slightly higher than  $I_{SC}$  or  $I_{Na}$  values reported for A6 cells reviewed in Helman and Kizer (1990) and Wills and Millinoff (1990), but lower than values reported by Bindels *et al.*, (1988). The  $I_{Na}$  aside, the mean value for  $i_{Na}$  averaging near 0.3 pA was similar to values from other studies (Wills and Zweifach, 1987; Granitzer *et al.* 1995), but slightly lower than values reported elsewhere (Helman and Kizer, 1990). The mean single channel conductance was calculated using the following equation:

$$\gamma = \frac{i_{Na}}{(V_m - E_{Na})} \quad (\text{eq. 8})$$

where

$$E_{Na} = 58 \log \frac{[Na]_o}{[Na]_i}$$

Substituting the mean  $i_{Na}$  of 0.31 pA (Table 1) and typical values for the appropriate variables: the  $\text{Na}^+$  extracellular  $\text{Na}^+$  concentration,  $[Na^+]_o$  was taken as 110 mM, the intracellular  $\text{Na}^+$  concentration as 14.5 mM (Els and Helman, 1991) and the membrane potential  $V_m$  as 71 mV (Koeppen *et al.*, 1983), we calculate an average  $\gamma$  of about 2.5 pS

which is consistent with values reported for the high selectivity, low conductance  $\text{Na}^+$  channel (Palmer and Frindt, 1986a).

$P_o$  averaged 0.3, again being remarkably similar to values determined in A6 cells by noise analysis (Granitzer *et al.*, 1995) and in cells of the rat CCT by patch clamp (Palmer and Frindt, 1986b). Accordingly,  $N_o$  and  $N_T$  averaged around 24 and 100 million channels/cm<sup>2</sup> respectively. The rate coefficients for the channels measured with CDPC were very similar to values reported previously (Helman and Kizer, 1990).  $k_{ob}$  averaged 7.63 rad/s.  $\mu\text{M}$ ,  $k_{bo}$  232.55 rad/s and  $K_B$  30.38  $\mu\text{M}$ . According to the values of the parameters for  $\text{Na}^+$  channels above, the properties of our  $\text{Na}^+$  channels were not significantly different from those reported in A6 cells or CCD from many other laboratories.

## 3.2 Effects of Aldosterone

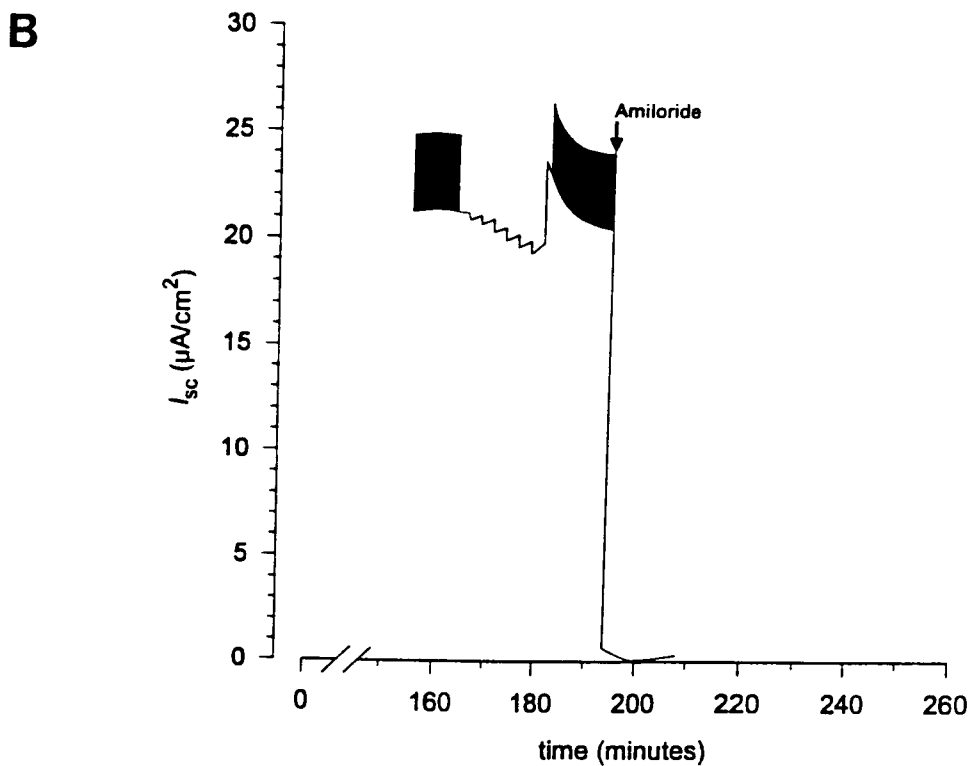
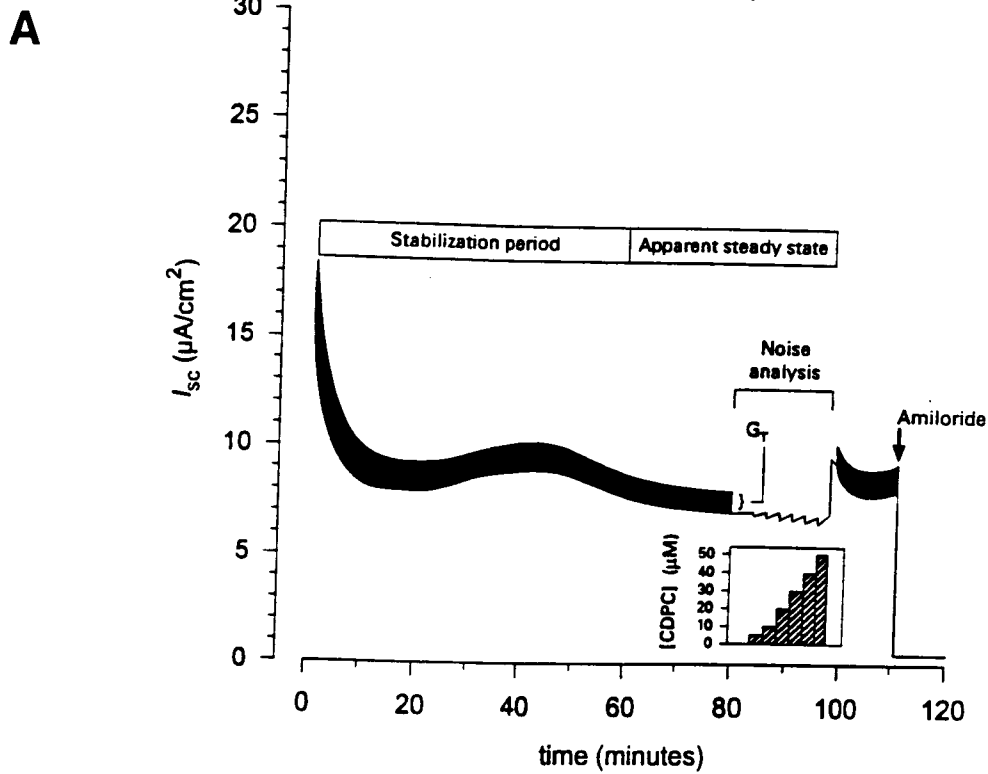
### 3.2.1 Steady State Data: Late Aldosterone Effects

The protocol for steady state experiments was used to examine the effects of exposing the cultured epithelia to aldosterone for periods of either 24 hours ( $n = 11$ ), 48 hours ( $n = 8$ ) or 72 hours ( $n = 3$ ) and compared to controls that had not been exposed to aldosterone as shown overleaf in Fig 3.2. Noise analyses were performed on paired control and experimental tissues on the same day (see Materials and Methods).

#### **Fig. 3.2 Steady state protocol of noise analysis**

*Shown above are the strip chart recordings of a typical paired steady state experiment which plots the  $I_{sc}$  as it varies with time throughout the experiment. A is the recording from control cells, while B is the recording from the paired experimental cells after exposure to 0.28  $\mu\text{M}$  aldosterone for 24 hours. Transepithelial conductances ( $G_T$ ) are indicated by the upward deflections of the current in response to an applied voltage (see Materials and Methods). Note the voltage generator was turned off during noise analyses. The histogram (inset) indicates the [CDPC] added in a stepwise manner producing the staircase pattern of inhibition of the  $I_{sc}$ . It can be clearly seen that aldosterone treated cells (B) displayed a larger transepithelial conductance*

and higher  $I_{sc}$  than the control cells (A). Addition of 100  $\mu\text{M}$  amiloride to the apical solution at the end of experiments caused an inhibition of the  $I_{sc}$  to near zero values in both A and B, hence nearly all the current was carried by  $\text{Na}^+$  ions. The  $I_{\text{Na}}$  was calculated from eq. 2. Notice the autoregulatory increases in the  $I_{sc}$  that occurred during noise analysis. This issue is discussed in greater detail in the section on the pulse inhibition protocol.



We decided to compare data from the 24 and 48 h experiments in order to determine whether there was any difference in the effects of aldosterone on the tissues after these times. The effects of aldosterone (24 hours) on the different parameters are illustrated in Fig. 3.1 (see also Fig. 2.5, for the PDS) and the mean values  $\pm$  S.E.M. for 24 h and 48 h experiments are summarised in Table 2 for convenience. Significantly, as can be seen from Fig. 3.1C, the slopes of the two rate concentration plots are the same and hence, exposure to aldosterone does not significantly alter the on-rate coefficient ( $k_{ob}$ ) of CDPC. Accordingly, all calculations of the values of individual parameters in the presence of aldosterone using the values of  $k_{ob}$ , are valid. The  $k_{bo}$  increased slightly ( $21.5 \pm 3.3$  %) in the 24 h and ( $9.8 \pm 3.6$  %) in the 48 h aldosterone experiments, which resulted in higher  $K_B$  values in the experimental tissues than the control (Table 2). When examining the data in Table 2, it is clear that both control and experimental tissues from the 24 hour experiments, were transporting at higher rates than their counterparts from the 48 hour experiments. Since many of the constituents of FCS (especially corticoids) are known to have an influence on the transport properties of cultured cells, lower transport rates might be expected in tissues used for the 48 hour experiments as they were bathed with serum free medium for longer periods than the 24 hour tissues (see Fig. 2.2). Importantly, despite these initial differences, exposure to aldosterone for either 24 or 48 hours brought about very similar percentage changes in the transport properties of the two groups as seen by the E/C values (Table 2) and Fig. 3.3.

As expected, aldosterone significantly increased the mean  $I_{Na}$  nearly 200 % above control levels after 24 and 48 hours (Fig. 3.1A and Table 2). The increase in  $I_{Na}$  occurred despite a decrease in  $i_{Na}$  of around 20 % (Fig. 3.1D). Consistent with the idea that hormones do not exert major effects on the single channel conductance,  $\gamma$  did not change significantly after 24 h aldosterone treatment, decreasing slightly from 2.9 in control to 2.54 in aldosterone as calculated above (Control Baseline Values). The decrease in  $i_{Na}$  was not due to a change

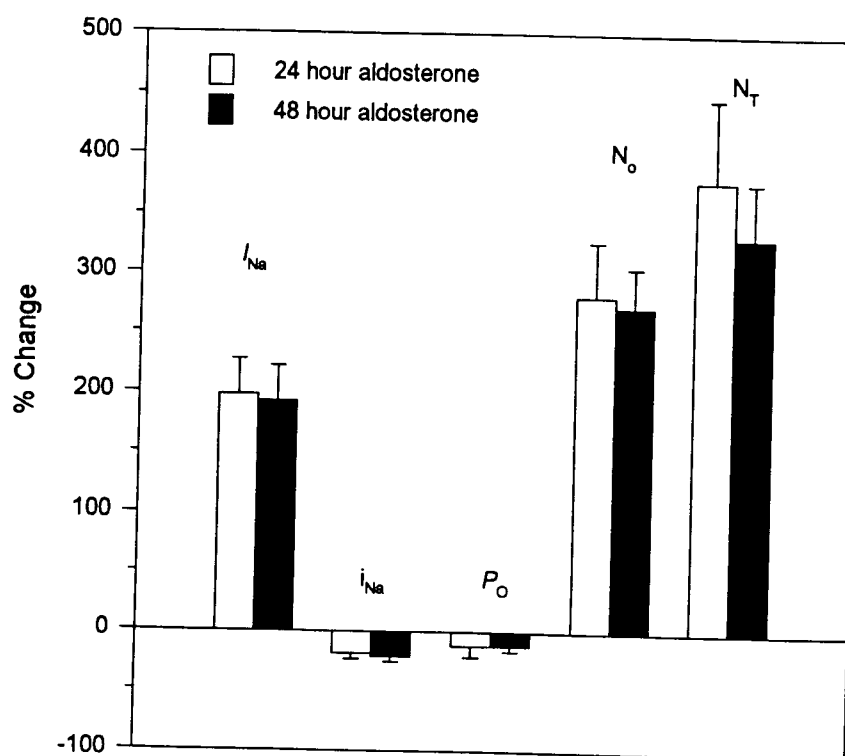
in channel conductance, but most probably was the result of a slight depolarisation of the apical membrane voltage (Koeppen *et al.*, 1983) leading to a decrease in the electrochemical driving force at the apical channels.

**Table 2: Effect of 24 and 48 hour aldosterone exposure on Na<sup>+</sup> channel properties.**

	<u>Hours</u>	<u>Control</u>	<u>Experiment</u>	<u>E/C</u>
$I_{Na}$ ( $\mu A/cm^2$ )	24 h	7.26 $\pm$ 0.62	20.13 $\pm$ 1.23	2.979 $\pm$ 0.295
	48 h	4.27 $\pm$ 0.61	11.70 $\pm$ 1.52	2.923 $\pm$ 0.297
$i_{Na}$ (pA)	24 h	0.36 $\pm$ 0.02	0.29 $\pm$ 0.02	0.813 $\pm$ 0.049
	48 h	0.29 $\pm$ 0.01	0.23 $\pm$ 0.01	0.789 $\pm$ 0.045
$P_o$	24 h	0.30 $\pm$ 0.03	0.25 $\pm$ 0.03	0.881 $\pm$ 0.092 *
	48 h	0.36 $\pm$ 0.04	0.32 $\pm$ 0.04	0.881 $\pm$ 0.044
$N_o$ ( $10^6/cm^2$ )	24 h	20.33 $\pm$ 1.69	71.45 $\pm$ 5.13	3.812 $\pm$ 0.457
	48 h	14.44 $\pm$ 2.03	52.23 $\pm$ 7.88	3.720 $\pm$ 0.335
$N_T$ ( $10^6/cm^2$ )	24 h	73.0 $\pm$ 8.5	327.0 $\pm$ 49.9	4.792 $\pm$ 0.679
	48 h	49.7 $\pm$ 12.5	201.4 $\pm$ 54.0	4.316 $\pm$ 0.460
$k_{ob}$ (rad/s. $\mu M$ )	24 h	7.94 $\pm$ 0.25	7.91 $\pm$ 0.25	1.002 $\pm$ 0.034 *
	48 h	7.24 $\pm$ 0.42	7.18 $\pm$ 0.33	0.997 $\pm$ 0.024 *
$k_{bo}$ (rad/s)	24 h	276.9 $\pm$ 17.9	334.7 $\pm$ 20.3	1.215 $\pm$ 0.033
	48 h	191.5 $\pm$ 12.7	211.0 $\pm$ 18.5	1.098 $\pm$ 0.036 *
$K_B$ ( $\mu M$ )	24 h	34.79 $\pm$ 1.82	42.47 $\pm$ 2.50	1.229 $\pm$ 0.064
	48 h	26.71 $\pm$ 1.69	29.70 $\pm$ 2.66	1.107 $\pm$ 0.047 *

*Table 2 is a summary of the effects of 24 (n = 11) and 48 hour (n = 8) aldosterone (0.28  $\mu M$ ) treatment on epithelial Na<sup>+</sup> channels. Control and experimental values are from paired experiments as explained in the text. Values at 0  $\mu M$  CDPC are given as means  $\pm$  S.E.M.. The fractional E/C values are produced by dividing the experimental value by its paired control value. This gives an idea of the fractional change in parameters from their control values with an E/C value of 1 indicating no change. The students t test was performed on paired data and non-significant changes ( $p > 0.05$ ) are indicated with \*.*

Importantly, the large increases in  $I_{Na}$  in both sets of experiments were clearly not due to changes in  $P_o$  which actually decreased slightly by about 20 %, being non-significant after 24 hours and just significant ( $p = 0.035$ ) after 48 hours. Hence, the increases in transport rates were mainly due to large changes in  $N_o$ , which increased significantly by about 275 % from control levels near 20 million channels/cm<sup>2</sup> (Fig. 3.1E,F and Table 2). This increase in  $N_o$  also compensated for the decrease in  $i_{Na}$ . Since  $P_o$  was not increased by aldosterone, it became clear that changes in  $N_o$  occurred by similarly large changes in  $N_T$  which increased by about 380 % and 330 % after 24 and 48 hours exposure to aldosterone respectively. Fig. 3.3 provides a graphical summary of the effects on the Na<sup>+</sup> channel parameters of 24 and 48 hours exposure to 0.28  $\mu$ M aldosterone.



**Fig. 3.3 Effects of aldosterone on Na<sup>+</sup> channel parameters**

A graphical illustration and summary of the steady state data comparing the mean % changes  $\pm$  S.E.M. of Na<sup>+</sup> channel parameters after 24 hour ( $n = 11$ ) and 48 hour ( $n = 8$ ) exposure to 0.28  $\mu$ M aldosterone.

It is of interest to note by examining Fig. 3.3 that changes to channel parameters ( $E/C$ ) due to 24 hour exposure to aldosterone were not significantly different from those as a result of 48 hour exposure. We also performed 3 experiments to examine the effects of stimulating the cells with aldosterone for 72 hours, but the results were not significantly different from either the 24 or 48 hour experiments and so we noted this, but did not pursue these experiments further. Hence, stimulating the tissues with aldosterone for 24, 48 or 72 hours induced similar changes in channel parameters which dictate the membrane permeability and ultimately the  $\text{Na}^+$  transport rate. It appears, at least from 24 to 72 hours, that the effects of aldosterone had reached a plateau. In light of this we decided to examine the earlier effects of aldosterone on tissues from time 0 to 12 hours after addition of the hormone.

### **3.2.2 Pulse Inhibition Protocol Data: Earlier Aldosterone Effects**

The pulse inhibition method of noise analysis allowed us to examine the time-course of changes to the properties of the  $\text{Na}^+$  channels by aldosterone continuously and during transient stimulation of the  $\text{Na}^+$  transport rate. An example of a strip chart recording plotted during a typical experiment following the pulse inhibition protocol is shown overleaf in Fig. 3.4.

#### **Fig. 3.4 Pulse inhibition protocol of noise analysis**

*A strip chart recording showing the results of a typical experiment using the pulse inhibition protocol. After an initial stabilisation period, tissues were continuously perfused with 10  $\mu\text{M}$  CDPC in the mucosal solution (indicated) and were again allowed to stabilise for about 2½ hours. Thereafter, at every 25 minutes, noise analyses were performed in the presence of 10  $\mu\text{M}$  CDPC and also after maximum  $I_{\text{SC}}$  inhibition following a pulse with 30  $\mu\text{M}$  CDPC. The effects of 0.28  $\mu\text{M}$  aldosterone (added to the basolateral medium at time 0) on the  $\text{Na}^+$  transporting properties of tissues were continuously monitored for 6 to 12 hours. Control periods and the addition of aldosterone are indicated on the graph. As before, the  $G_T$  could be determined from the resultant change in  $I_{\text{SC}}$  due to an applied voltage, which was always switched off during noise analyses. 100  $\mu\text{M}$  amiloride was added to the apical medium at the end of the experiment to determine the  $I_{\text{Na}}$  as before (eq. 2).*

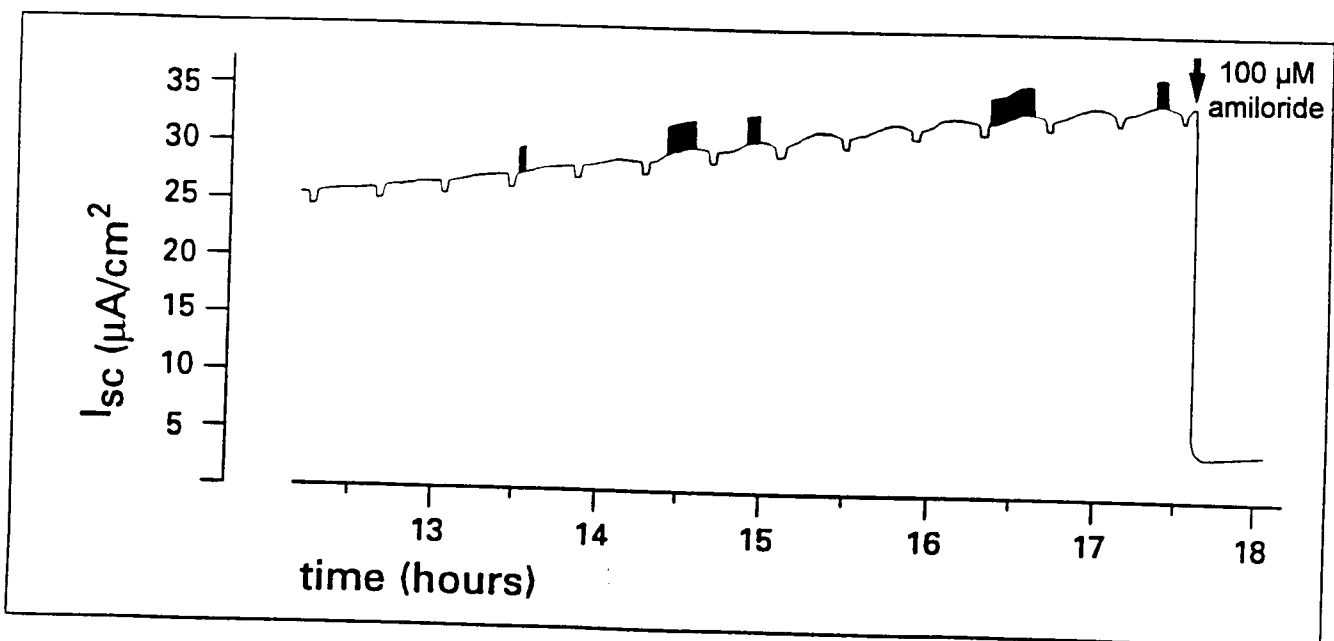
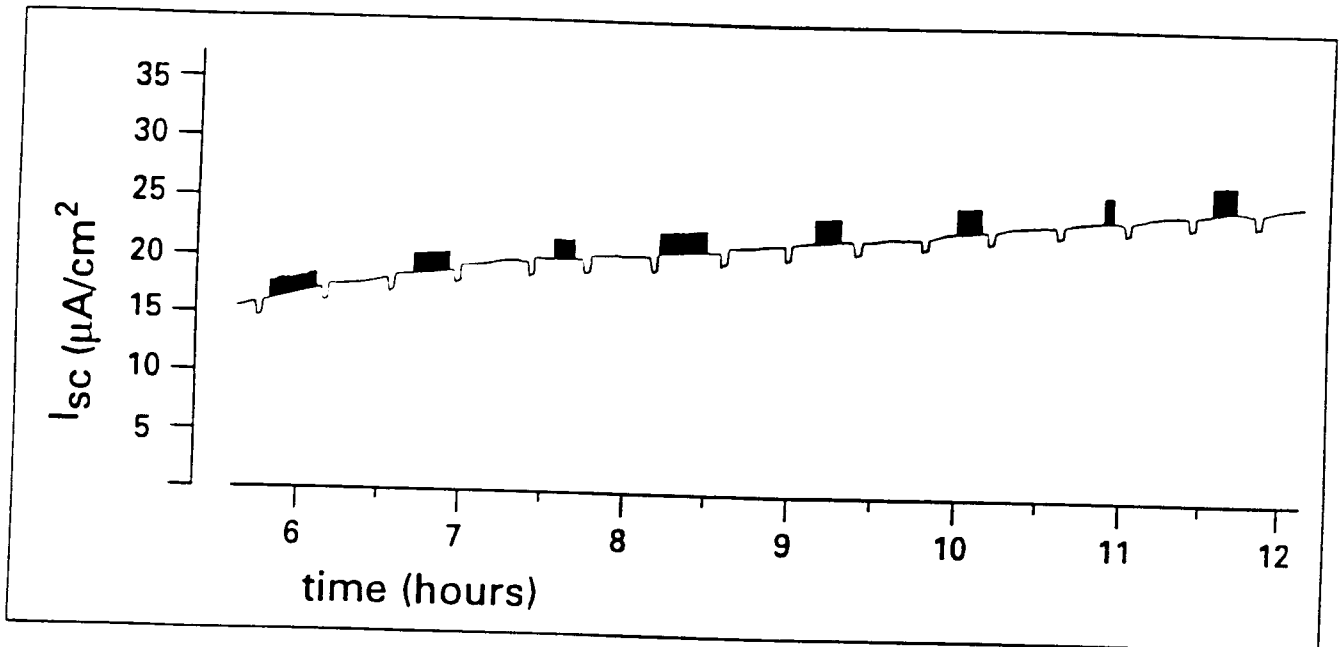
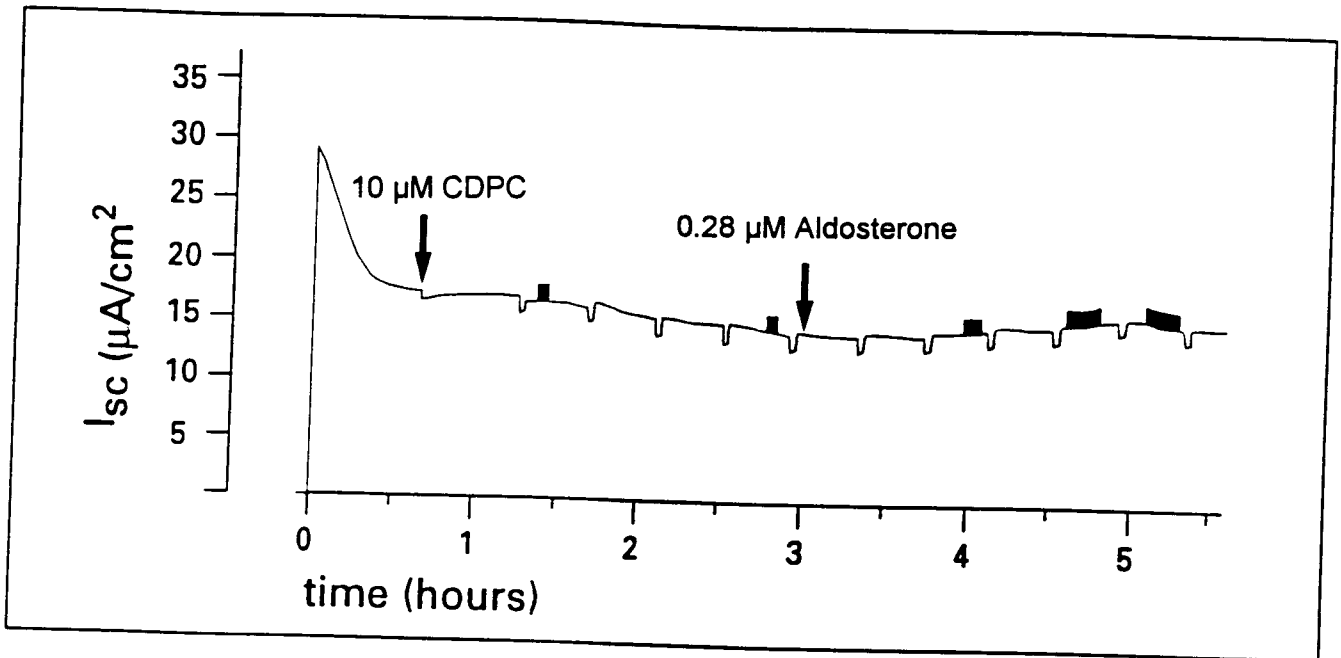
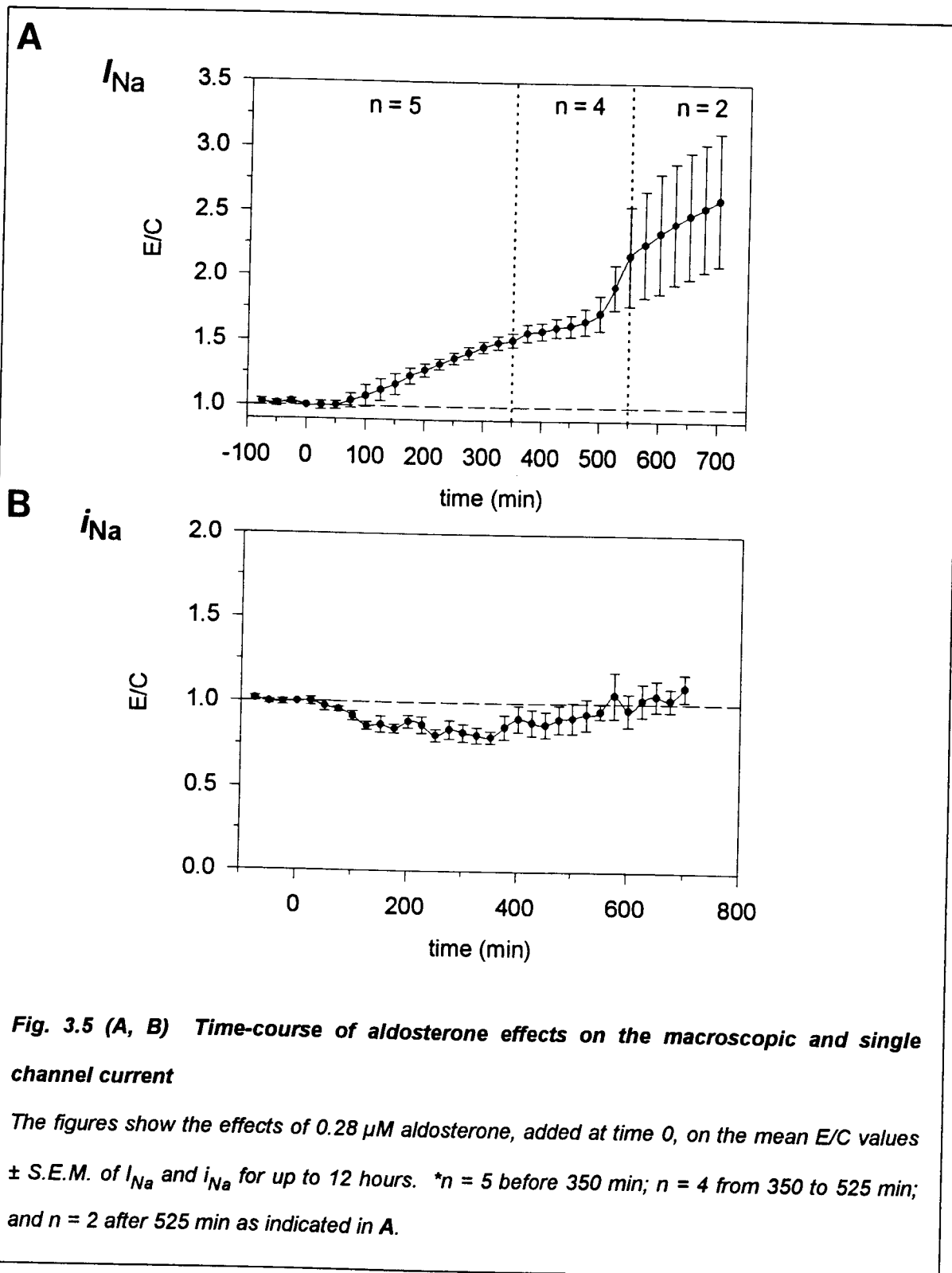


Fig. 3.4 illustrates the typical time-course of changes in the  $I_{SC}$  caused by aldosterone over many hours. As can be seen, the addition of aldosterone to the basolateral medium of A6 cells at time zero, caused a significant increase in the  $I_{SC}$  first seen after a lag period of approximately 2 hours. Stimulation of the  $I_{SC}$  continued and after 12 hours, the  $I_{SC}$  had doubled from a control value of  $15.96 \mu\text{A}/\text{cm}^2$  to  $32.43 \mu\text{A}/\text{cm}^2$ .  $I_{SC}$  increases were the result of increases in  $G_T$  which, after 12 hours, had increased 66 % above control levels.

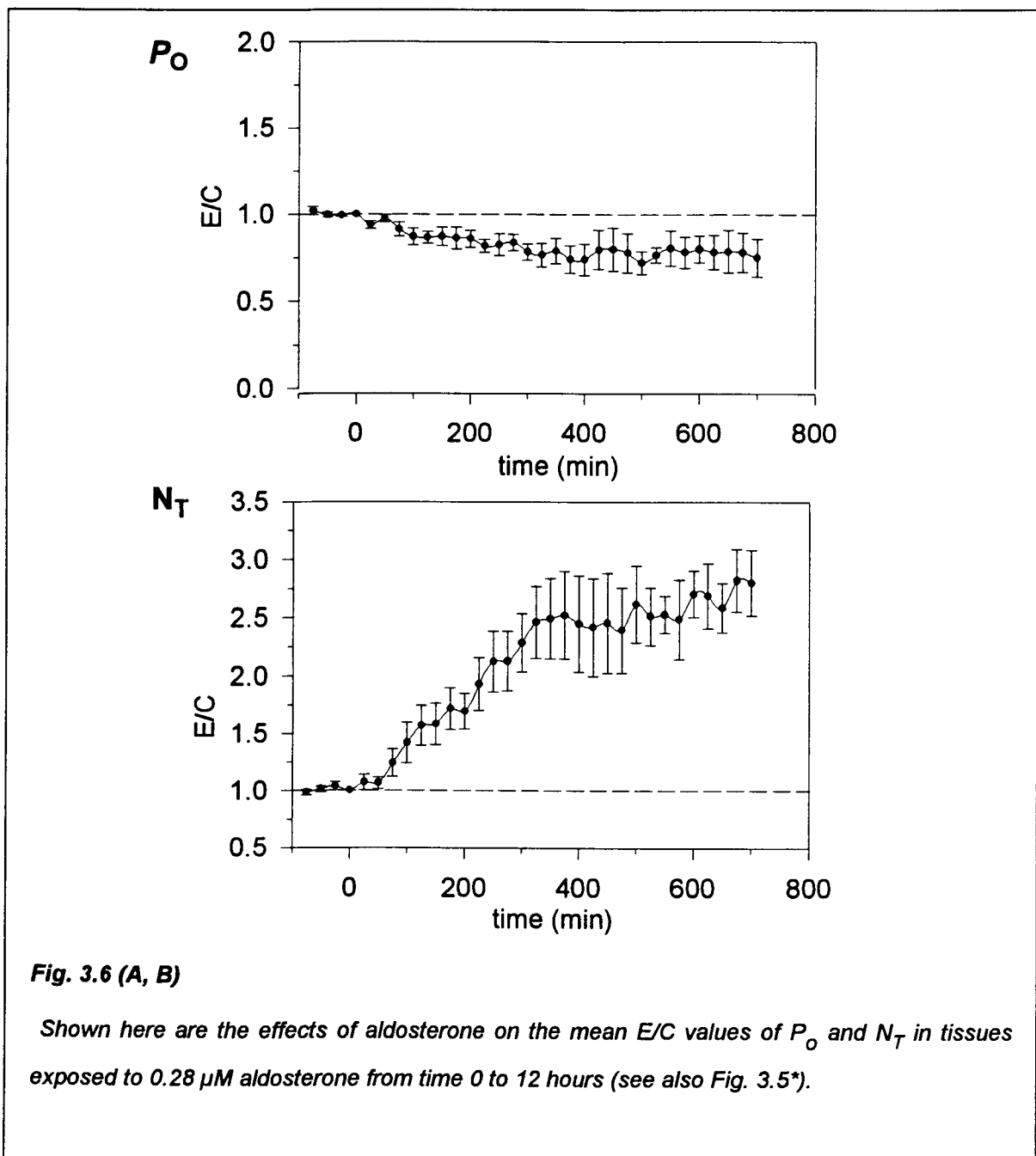
The mean  $I_{Na}$  value recorded during control periods of the pulse inhibition experiments was significantly higher than those of the steady state controls (see Table 2). The  $V_T$ 's of these inserts were also high, especially the last three which averaged  $58.2 \pm 8.17 \text{ mV}$  compared to the group as a whole which averaged  $38.54 \pm 12.98 \text{ mV}$ . A factor that could have resulted in the higher transport rates and  $V_T$ 's is that a freshly made batch of medium was used during the growth of inserts for these experiments. It was observed that lower  $V_T$ 's and  $I_{SC}$ 's were obtained from cells bathed with medium that had been made up for more than a month (data not shown). It has been suggested that this could be due to the breakdown of L-glutamine in the medium, which is a source of energy for the cells. L-glutamine can be replaced in the medium at  $\frac{1}{2}$  the original concentration after 3 weeks, but seeing as the original concentration was high (584 mg/l) it was decided not to replenish this component. The high levels of L-glutamine in the newly made up batch of medium could have been responsible for the higher voltages and currents recorded from these monolayers. Another factor of note is that these three inserts were older than the others in the group and those of the steady state experiments and they possibly had formed tighter more polarised epithelial monolayers, which resulted in the higher voltages and currents.

The changes to the  $\text{Na}^+$  channel parameters caused by aldosterone and underlying the increase in  $\text{Na}^+$  transport rate (Fig. 3.4) are illustrated in Fig. 3.5, 3.6 and 3.7.

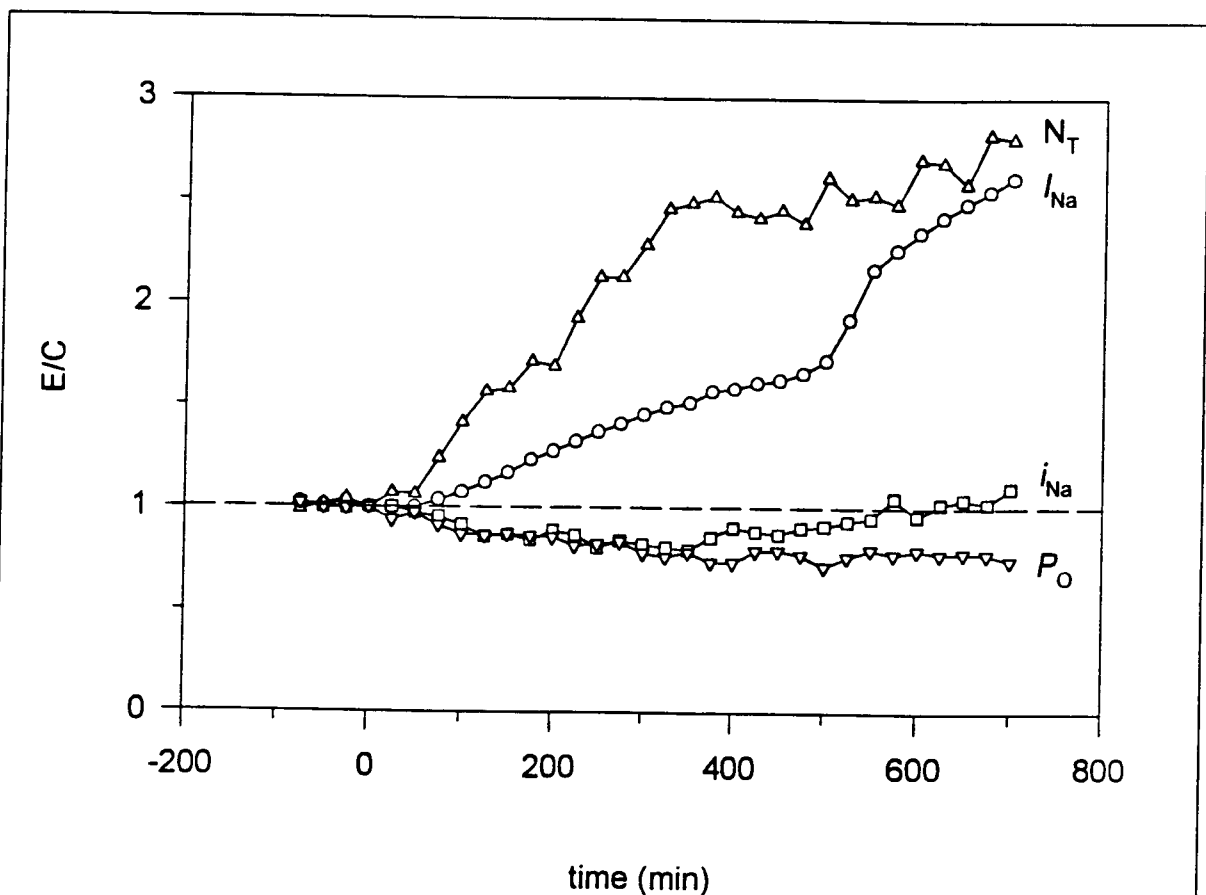


The time course of changes to the  $I_{\text{SC}}$ , as the  $I_{\text{Na}}$ , are illustrated in Fig. 3.5A. Aldosterone caused increases in  $I_{\text{Na}}$  (Fig. 3.5A) that followed the same time course as changes in  $I_{\text{SC}}$ . It appears that there may be a biphasic response of  $I_{\text{Na}}$  to aldosterone, since there appears to be a steeper increase in  $I_{\text{Na}}$  from about 8 hours after the exposure to the hormone. This

could correspond to the late phase of the transport response (see Literature Review). However, note that the error bars are much longer beyond this point since "n" has decreased to 2 and so more experiments would be necessary to validate such a response. As can be seen from Fig. 3.5B, the results using the pulse inhibition protocol, consistent with those of our steady state experiments, also indicate that aldosterone has little effect on the  $i_{Na}$ . After an initial small, but significant decrease in  $i_{Na}$  (around 20 % at 6 hours), the  $i_{Na}$  values returned to control levels after 12 hours. Thus any changes in the permeability of the apical membrane which results in increases in the  $Na^+$  transport rate, must be due to increases in either  $P_o$  or  $N_T$  (Fig. 3.6).



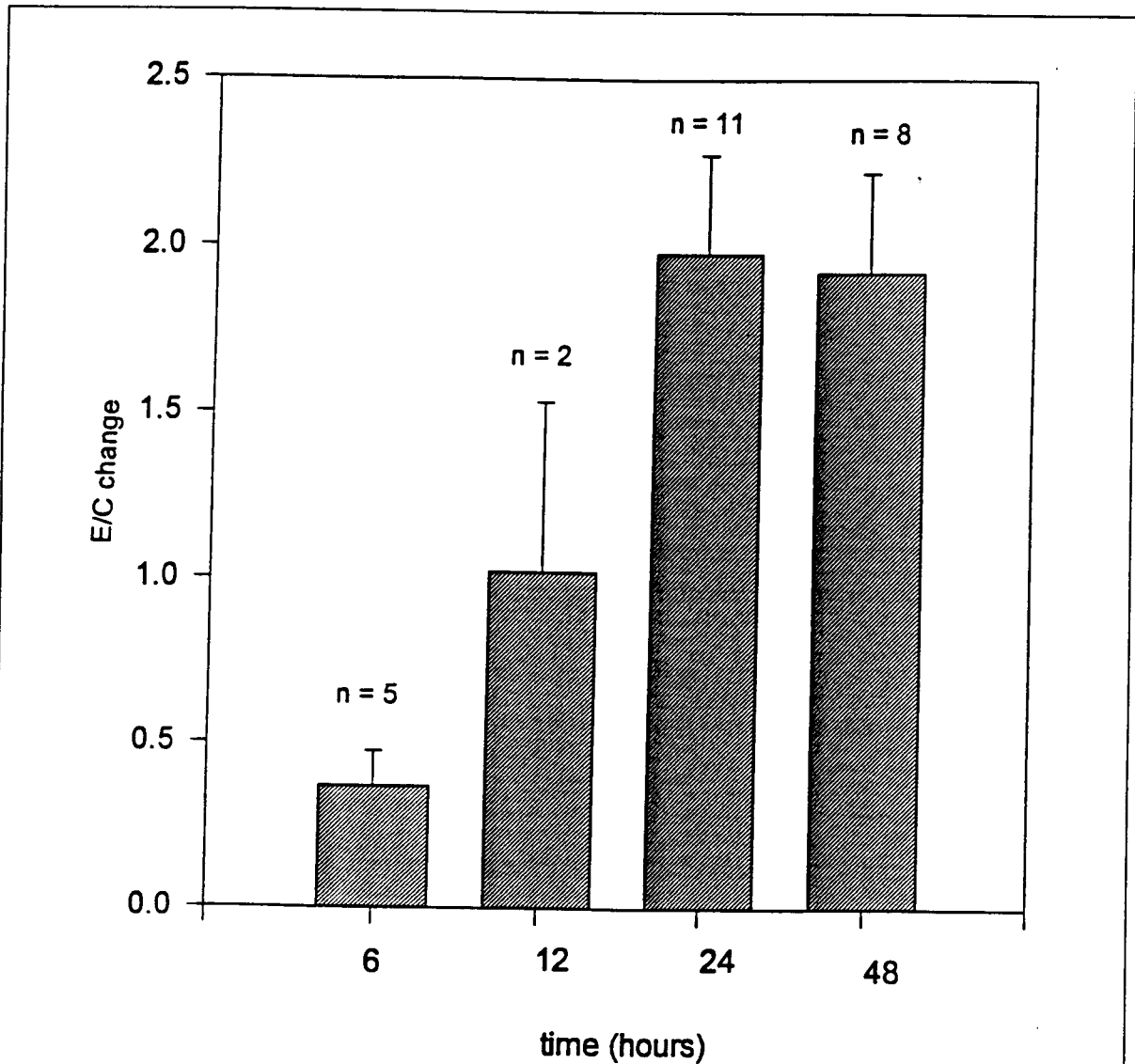
From Fig. 3.6A it is clear that  $P_o$  remained fairly constant for the first 4 hours after the addition of aldosterone, thereafter decreasing steadily below control values. Despite the decrease in  $P_o$  and  $i_{Na}$ , there were large increases in  $I_{Na}$  which were due mainly to parallel increases in  $N_T$  which followed a similar time course (Fig.3.7). 6 hours after the exposure to aldosterone, the  $N_T$  had increased above control levels by nearly 150 % and continued to increase over the remainder of the period studied, to almost 300 % after 12 hours (Fig. 3.6B). A graphical summary of the aldosterone-induced changes in  $Na^+$  channel parameters up to 12 hours is presented in Fig. 3.7.



**Fig. 3.7** A summary of the results from pulse inhibition experiments

This figure illustrates the changes in the mean E/C values of  $Na^+$  channel parameters up to 12 hours after the addition of  $0.28 \mu M$  aldosterone at time 0.

Fig. 3.8 is presented as a graphical summary of the effects of aldosterone on the  $\text{Na}^+$  transport rate. The graph is a compilation of our data gained from both the steady state experiments and the pulse inhibition protocol experiments. As illustrated here, after stimulation with aldosterone, the  $I_{\text{Na}}$  increased substantially with time reaching apparent steady state values after about 24 hours.



**Fig. 3.8 Summary of the effects of aldosterone on the  $\text{Na}^+$  transport rate**

This graph shows the aldosterone stimulated increases in  $I_{\text{Na}}$  combined from the results of the pulse inhibition experiments and the steady state experiments. Bars indicating mean E/C values for  $I_{\text{Na}}$ 's  $\pm$  S.E.M., are given at selected times. The number (n) of experiments at the selected times is indicated on the graph (see also Fig. 3.6\*)

## Chapter 4

# Discussion

---

---

### 4.1 Control Baseline Values

A6 cells that were grown on permeable inserts, formed tight epithelial monolayers that transported  $\text{Na}^+$  at expected rates. Given the wide variability in baseline levels of transport rate, already alluded to in the results, our baseline values are consistent with values presented elsewhere (Helman and Kizer, 1990). Single channel parameter values, determined by noise analysis, were comparable with data reported by most other laboratories. Significantly,  $P_o$  values, averaging around 0.3, were remarkably similar to values obtained for these and other epithelia with both noise analysis (Wills and Zweifach, 1987; Granitzer *et al.*, 1995) and patch clamp (Palmer and Frindt, 1986a, 1986b; Pácha *et al.*, 1993). Accordingly, the  $\text{Na}^+$  channels in the apical membranes of A6 cells, grown in our laboratory, were similar to the amiloride-sensitive,  $\text{Na}^+$ -specific, low conductance channel found in the rat CCD and elsewhere.

As clearly seen from the time course experiments, exposure of cultured A6 cells to  $0.28 \mu\text{M}$  aldosterone in the basolateral medium caused increases in  $I_{\text{SC}}$  which were first noticeable after a latent period of about 60 to 90 minutes. Similar latent periods ranging from 30 min to 120 min have been reported for various tight epithelia (Schafer and Hawk, 1992; Verrey, 1995). As already discussed in the introduction, it is thought that synthesis of the aldosterone-induced proteins responsible for the subsequent natriuretic effects of aldosterone occurs during this phase. Following the latent period, the  $I_{\text{SC}}$  increased steadily, reaching apparent maximum values after 24 hours and thereafter maintaining this level for the 48 and 72 hour periods of our studies. (We did not examine the effects of chronic exposure to aldosterone). Our values measured under steady state conditions after

24, 48 and 72 hours provide information on the later natriferic responses to aldosterone, while the continuous recordings of the time course experiments, provide new information on earlier natriferic responses.

## 4.2 Summary of the Steady State Data

The increase in  $I_{SC}$  produced by aldosterone after 24 hours is mainly the result of modulation of  $Na^+$  channels in the apical membrane. As shown, aldosterone does not exert these effects by changes in the single channel conductance, even though we did observe small decreases of  $i_{Na}$  (Fig 3.1 and Table 2). Instead, aldosterone's effects on the  $I_{SC}$  could have been mediated by changes to channel open probability and/or channel densities.

### 4.2.1 Aldosterone Does Not Stimulate Channel Activity by Increases in Open Probability

The main aim of this investigation was to determine whether aldosterone-induced effects on the  $Na^+$  transport rate are mediated via changes to  $P_o$  or  $N_T$ . This proposal was the result of conflicting data regarding the mechanisms of action of the hormone. Specifically, with patch clamp, Kemendy *et al.* (1992) first observed that depleting A6 cells of aldosterone for between 24 and 36 hours caused a decrease in the channel activity, or  $NP_o$  to 20 % of the control values. Readdition of the hormone to aldosterone-depleted cells caused a 200 % increase in  $NP_o$  when compared with cells from which aldosterone had not been removed. This was equivalent to a 1760 % increase over hormone-depleted cells. Amongst their observations was an apparent increase in channel number, estimated from the number of observable current levels in the patch recording. However, statistically, this was reported as non-significant and the increase in channel activity ( $NP_o$ ) was attributed instead, to increases in mean channel open time without a significant effect on the mean closed time. Hence  $P_o$  increased in those cells that were restimulated with aldosterone. The  $P_o$  in aldosterone-depleted cells was extremely low, averaging around  $0.04 \pm 0.02$  while 6 hours after aldosterone readdition, the mean  $P_o$  was  $0.38 \pm 0.04$ . This represents an increase in

$P_o$  of 950 %. Consequently, from these studies, it was proposed that aldosterone stimulates the  $\text{Na}^+$  transport rate by large increases in channel open probability and not by changes in channel density.

Our results were entirely different from this. In fact, the  $P_o$  values remained relatively unchanged after 24 hours, while there was a slight decrease in  $P_o$  of about 12 % ( $p < 0.35$ ) after 48 hours exposure to aldosterone. We cannot comment on the significance of effects after 72 hours because too few experiments were performed, but even in these cells aldosterone had little effect and the  $P_o$  remained close to control levels.

#### **4.2.2 Aldosterone Causes Large Increases in Channel Densities**

Consistent with the idea that aldosterone does not have major effects on the  $\text{Na}^+$  channel densities and, hence, apparently in agreement with the idea of major effects on  $P_o$ , were results mainly from immunocytochemical studies. Kleyman *et al.*, (1989) used an antibody directed against the amiloride binding site of  $\text{Na}^+$  channels and a photoactive amiloride analogue to demonstrate that the number of  $\text{Na}^+$  channel proteins are not significantly altered by aldosterone within 16 h. Results showing that aldosterone does not change the expression of  $\text{Na}^+$  channels at the membrane of A6 cells after 16 h (Kleyman *et al.*, 1992) was also used as support for the suggestion that aldosterone does not have a major effect on  $\text{Na}^+$  channel densities.

Not all data are consistent with these proposals. Results from earlier studies using amiloride-induced noise analysis suggested that aldosterone did not effect the  $P_o$  of the channels but, instead had main effects on the channel densities (Palmer *et al.*, 1982). This assumption was later supported by results from patch clamp studies (Pácha *et al.*, 1993). They increased the plasma aldosterone levels in rats by placing them on a low  $\text{Na}^+$  diet for 12 h. When cells from the CCD of rats treated in this way were studied, an increase to the mean number of open channels per patch was reported, compared to CCD of control rats

that had not been placed on the low- $\text{Na}^+$  diet. Specifically data showed that this change was not accompanied by any significant effect to the  $P_o$  of  $\text{Na}^+$  channels. Accordingly, this left open the probability that, contrary to the conclusion by Kemendy *et al.* (1992)<sup>3</sup>, aldosterone may have major effects on the regulation of  $\text{Na}^+$  channel densities. Our results clearly supported this theory. Instead of modulating  $P_o$ , aldosterone caused large increases (by approximately 280 %) in the number of open channels which increased on average from around 20 to 70 million channels/cm<sup>2</sup> after 24 hours exposure to aldosterone. Similar increases occurred after 48 and 72 hours (Table 2).

Increases in the number of open  $\text{Na}^+$  channels in the apical membrane may occur by either increasing the open probability ( $P_o$ ) of conducting channels, or by increases to the total number ( $N_T$ ) of active channels in the membrane. Since the  $P_o$  of  $\text{Na}^+$  channels did not increase over the periods of the study, the increase in the number of open channels had to occur by an expansion to the total number of active channels in the membrane ( $N_T$ ). Our data, from both the steady state experiments and the time-course experiments, show that aldosterone augments the  $I_{SC}$  nearly three fold, primarily through large increases in  $N_T$  (over 400 %) that follow a similar time course as to the  $I_{SC}$ . These results are in agreement with earlier data reported from studies that examined the effects of aldosterone on frog lung epithelium and the effects of glucocorticoids on A6 cells (Fischer and Clauss, 1990; Granitzer *et al.*, 1995).

We have no idea why our results are so different from those of Kemendy *et al.* (1992), but would like to consider the following issues.

---

<sup>3</sup>After 6 h readmission of aldosterone, Kemendy *et al.* (1992) observed an increase in the number of open channels beneath the patch but believe that this is not significant. However, if you compare values of the aldosterone-treated cells with those of values of aldosterone-depleted cells, as they did to calculate changes to  $P_o$ , then there is a huge increase (around 500 %) in the mean observed number of current levels (Table 2, p 829). Accordingly, aldosterone may have affected  $\text{Na}^+$  channel densities.

### **4.2.3 Speculations on the reasons for the differences in results**

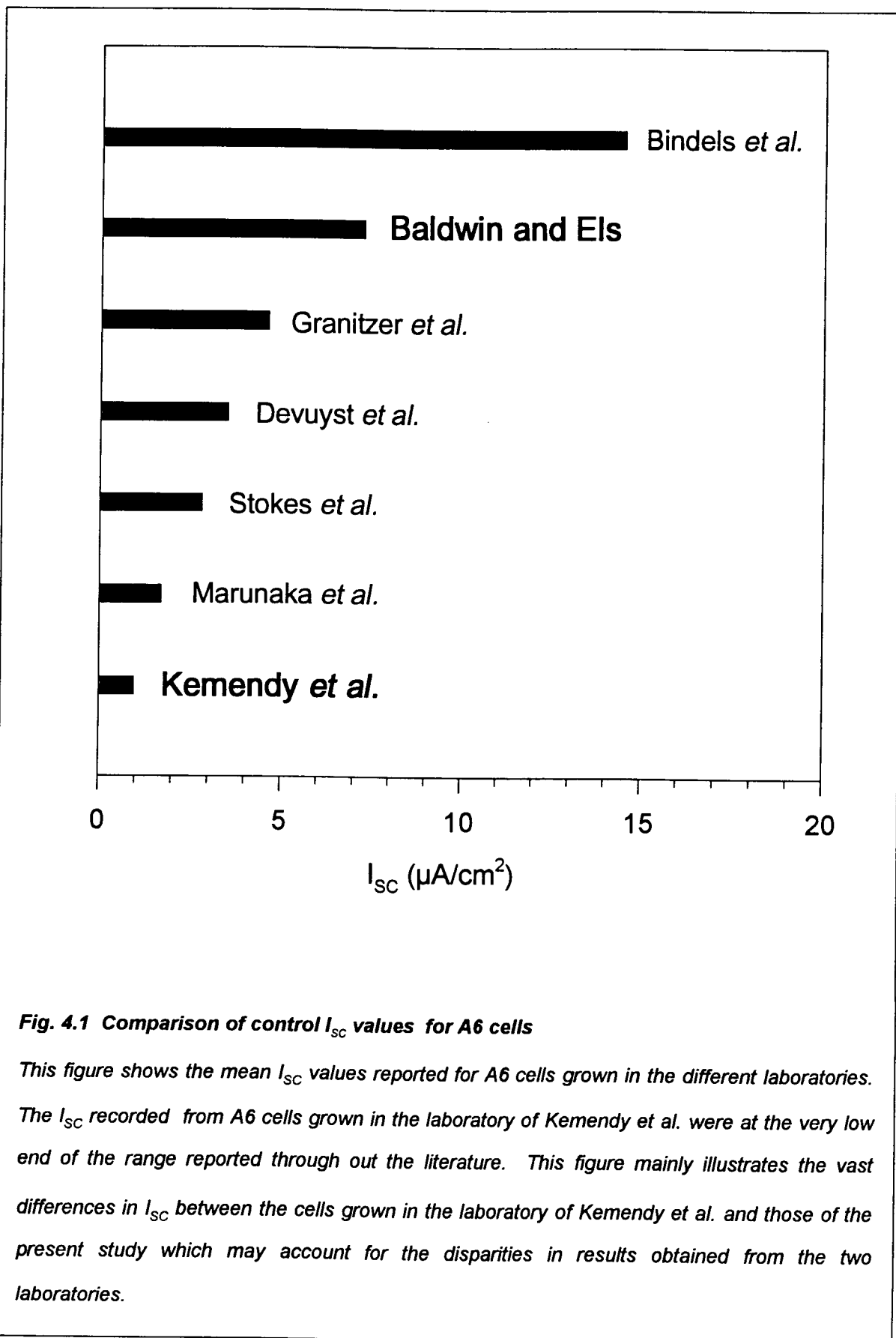
Direct comparisons of our data and those by Kemendy *et al.* (1992) are difficult because of a number of differences in the preparations and protocols. Regardless, the final results should be very similar. Generally, disparities in results from different laboratories may be attributed to differences in methods or differences in experimental procedures and/or tissues. From past experience, data obtained using either patch clamp or noise analyses are similar (see above). Specifically, other data obtained by these two methods in the two laboratories concerned are compatible, indicating that AVP causes an increase in the  $\text{Na}^+$  transport rate by increases in channel densities and without an effect on  $P_o$  (Els and Helman, 1991; Marunaka and Eaton, 1991). Hence, we would not expect that differences in methods would be the main cause of the differences in the present results.

There were large differences in the way in which cells were cultured and in the manner in which the experiments were carried out between the two studies, making direct comparisons difficult. Although we use a different clone of A6 cell, we are not aware of any differences that this might cause. We performed our experiments on cells that had been grown for periods in excess of 10 days whereas Kemendy *et al.* (1992) performed their experiments on younger cells (usually 9 days old). The reasons why we selected tissues that were older than 10 days was because, as shown in Materials and Methods (see also Fig. 2.1) by this age they have developed significantly large transepithelial voltages and rates of sodium transport. Interestingly, it has recently been observed in our laboratory that adenylate cyclase does not appear in the basal membranes of A6 cells before about 10 days (Els and Butterworth, personal communication). Hence the cells might not be properly polarized before that time and certain components involved in channel regulation may not be present.

#### 4.2.3.1 Differences in Sodium transport rates

The most important difference between our studies was the manner in which the tissues were prepared. Kemendy *et al.* (1992) found it necessary to grow their cells in the presence of aldosterone and, hence, could not show the direct effect of the addition of aldosterone addition to non-stimulated cells. The main reason for doing this was that they could only record channel activity when cells had been grown to maturity (9 days) and only in the continuous presence of aldosterone. We clearly did not experience this difficulty and we could perform experiments on cells cultured without aldosterone and without any FCS for 48 h since these cells had substantial rates of Na<sup>+</sup> transport. This is in agreement with other studies where cells were grown in the absence of aldosterone and presented substantial rates of Na<sup>+</sup> transport (Wills *et al.*, 1993). In fact, the disparate rates of Na<sup>+</sup> transport was a major difference between the two studies. Our mean  $I_{sc}$  of control tissues, in the absence of FCS for 48 h, was  $4.43 \pm 0.63 \mu\text{A}/\text{cm}^2$  (and slightly higher in the 24 h control group). This value was substantially higher than the mean  $0.91 \mu\text{A}/\text{cm}^2$  reported by Kemendy *et al.* (1992) for their cells which had been depleted of aldosterone for 50 h. In fact, as illustrated in Fig. 4.1, when compared to others, the cells used in their study had the lowest baseline rate of Na<sup>+</sup> transport, making comparisons complicated. The reason for this difference is not known, but it may be due to the fact that they grew their cells on collagen coated plastic inserts and not on porous cellulose filters. Cells grown on non-porous supports have completely different characteristics (c.f. Sariban-Sohraby *et al.*, 1995).

It is of interest to note that the  $P_o$  of the channels depleted of aldosterone for 72 h only averaged 0.04. This value is extremely low and similar ones have not been reported anywhere throughout the literature, with the exception of channels that were isolated and then reconstituted into lipid bilayers (Ismailov *et al.*, 1994).



**Fig. 4.1 Comparison of control  $I_{SC}$  values for A6 cells**

This figure shows the mean  $I_{SC}$  values reported for A6 cells grown in the different laboratories. The  $I_{SC}$  recorded from A6 cells grown in the laboratory of Kemendy et al. were at the very low end of the range reported through out the literature. This figure mainly illustrates the vast differences in  $I_{SC}$  between the cells grown in the laboratory of Kemendy et al. and those of the present study which may account for the disparities in results obtained from the two laboratories.

#### 4.2.3.2 Are We Dealing with the Same Sodium channel?

Reasons for this low  $P_o$  are not known. It might be that they are dealing with a different channel type in their experiments. Kemendy *et al.* (1992) have, in fact, reported that 80 hours after the removal of aldosterone, the low conductance, high selectivity channel (as identified in our tissues) are only seen infrequently and instead different, higher conductance 10 pS channels, with low  $\text{Na}^+$  to  $\text{K}^+$  selectivity become more prevalent. These high conductance channels have not been reported in native epithelia, but their occurrence is limited to cultured cells grown on non-porous supports and in poorly  $\text{Na}^+$  transporting cultures (for a review see Garty, 1994). The low conductance channels may also degrade with time to a lower selective higher conductive channel (reviewed by Lewis and Donaldson, 1990; Wills and Zweifach, 1987).

While suggesting that aldosterone does not modulate channel densities and that its main action is to increase the channel open probability (Kemendy *et al.*, 1992), a recent review from the same laboratory suggested a slightly different interpretation of the data (Eaton *et al.*, 1995). They suggested that in the original study aldosterone apparently increased the  $P_o$ , not of conducting channels, but of a different channel sub-type, referred to as "cryptic" or non-conducting channels with  $P_o$  values equal to, or very close to zero. The presence of such non-conducting channels can not be detected using any electrophysiological technique since such channels would be electrically silent and hence would not contribute to the channel activity. This may provide an explanation for the curious observation that they were unable to observe any channel activity in the membranes in the absence of aldosterone. Provided aldosterone does cause an activation of cryptic channels, this would clearly present as an increase in the total number of activated channels. This increase would easily be measured with noise analysis as an increase in  $N_T$ , consistent with our data. These presumptions will remain unresolved until the existence and behaviour of quiescent channels has been determined.

#### 4.2.3.3 Effects of osmolarity

Another issue that may be considered has to do with the osmolarity of the solutions. The transport rate of cells is greatly affected by osmolarity changes (Wills *et al.*, 1991). Changing the solutions bathing A6 cells from an iso-osmotic (200 mOsm/l) to a hyper-osmotic solution (290 mOsm/l), was responsible for large decreases in the  $I_{sc}$ , decreasing from an average of  $25 \mu\text{A}/\text{cm}^2$  to  $0.6 \mu\text{A}/\text{cm}^2$  by decreases in the channel activity. The osmolarity of the bathing solutions (approximately 300 mOsm/l) used by Kemendy *et al.* (1992) were even higher than those used by Wills *et al.* for their experiments. Hence, a change from growth medium, usually between 200 and 250 mOsm/l, to high osmolarity bathing solutions could certainly have caused effects to the channels and resulting in the low baseline transport rates and low  $P_o$  of the channels. We have specifically taken cognisance of this fact and avoided the problems of any osmolarity changes by using the same growth medium (about 250 mOsm/l) to perfuse the cells in the chambers.

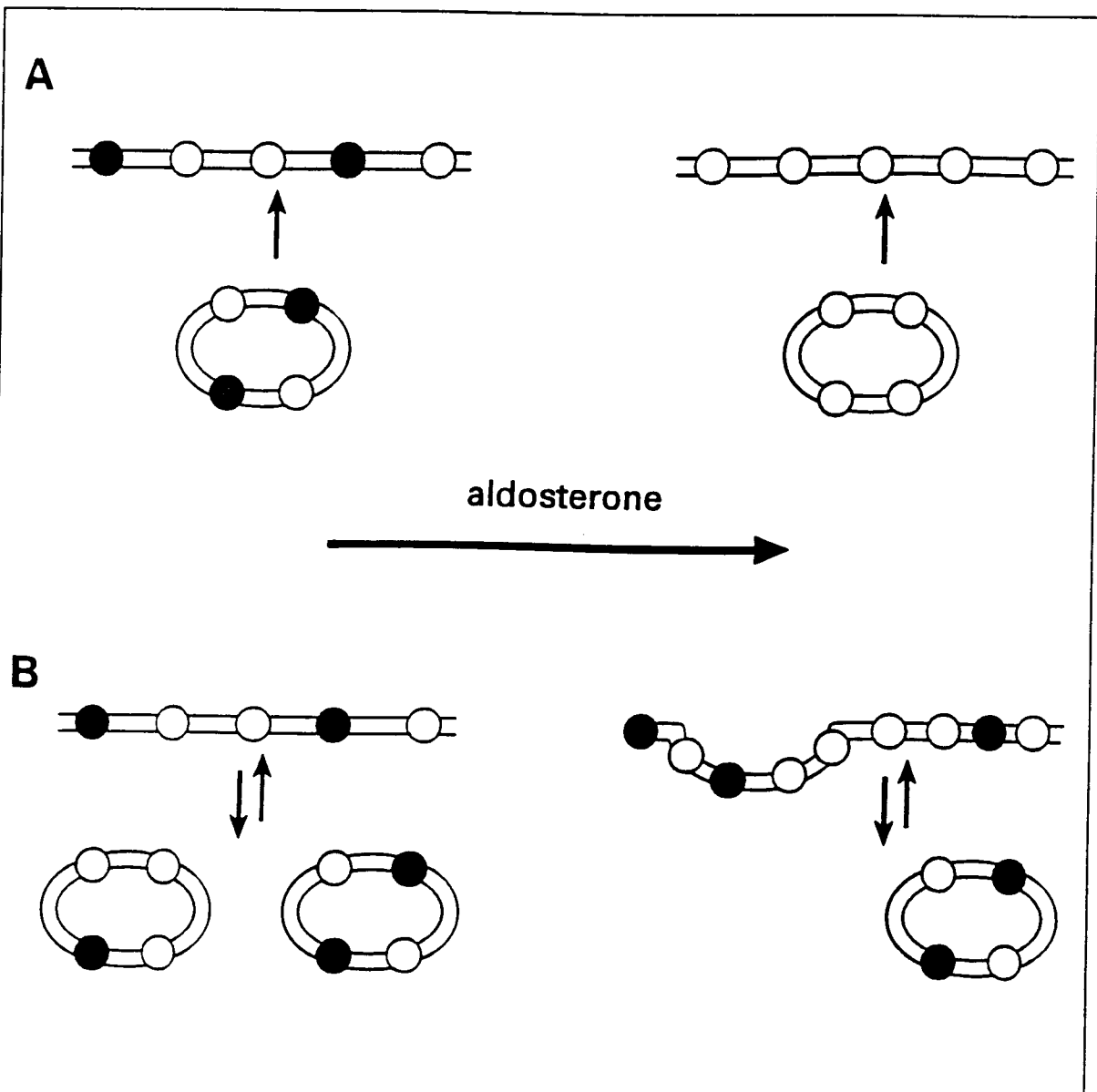
### 4.3 Comments on Possible Mechanism of Aldosterone Action on Sodium Transport

Examination of our data from the time course experiments indicated that aldosterone caused significant increases in the  $I_{sc}$  only after a lag phase of about 60 to 90 minutes. With the pulse inhibition protocol method of noise analysis, we determined that during this latent period, aldosterone had no significant effects on the  $\text{Na}^+$  channels. This period probably coincides with the synthesis of AIP's by aldosterone action. The latent phase was followed by a period of about 5 h during which the  $I_{sc}$  increased rapidly. The rates of transport were then maintained for the duration of the experiments (about 12 h). The early natriferic phase of the hormone was characterised by large increases of  $N_T$  (Fig. 3.6B). These increases in channel densities also compensated for the gradual and limited decrease in  $i_{\text{Na}}$ , which was probably due to a small depolarisation of the apical membrane

voltage. Consistent with what we found with our steady-state protocols, there was no significant change in  $P_o$  during this period.

Our data does not favour any of the theories to explain mechanisms whereby changes in channels are brought about. Results from other studies seemingly favour the idea that there is an activation of channels in the membrane during the early natriphic phase caused by aldosterone. We have at least demonstrated that this activation does not occur by altering the gating characteristics of the channels but rather by increases in the total number of active channels in the membrane. This is consistent with the model described by Schafer and Hawk (1992) leaving open the possibility that active channels may be recruited from the cytoplasm (also refer to the diagram in Fig. 4.2).

Unfortunately, many studies investigating the question of channel insertion only examined the early effects of the hormone (Garty and Edelman, 1983; Sariban-Sohraby *et al.*, 1995; Kleyman *et al.*, 1989). Evidence presented by Asher and Garty (1992) indicated that new channels are synthesised within 20 h of addition of aldosterone. Nevertheless, there is evidence supporting the idea that channels may be inserted in some way as a result of the action of aldosterone, especially so later, during the natriphic action of the steroid. Preliminary evidence from impedance analysis indicated that aldosterone increases the apical membrane capacitance after 18-24 h in aldosterone, concomitant to the increase in transport rate (Liu *et al.*, 1995). The changes were especially noticeable in a relaxation domain believed to be attributable to  $\text{Na}^+$  channels and hence the data support the idea that increases in channel density by aldosterone are mediated by vesicle trafficking of channels between the cytoplasm and the apical membrane. However, the apical membrane of A6 cells are heterogeneous with regard to their domains of dielectric behaviour and many more analyses have to be performed to determine the contribution of each domain or dielectric relaxation process before the analysis of the data may be rigorously interpreted. Immunocytochemical studies have been unable to provide



**Fig. 4.2 Suggested models for the action of aldosterone**

Quiescent channels present in the membrane (solid circles) may be directly activated by aldosterone (open circles) especially so during the early natriphic phase. Also shown in A, is the possibility that new channels may be gained (but not recycled) from the cytoplasm. It is not known whether these are quiescent or active forms of the channel. In B we show the idea that channels may be gained by regulated recycling of channels at the apical membrane. These channels may be either active or quiescent. It appears that in the late natriphic phase of aldosterone action there is a production of new channels resulting in an enlargement of the total pool of channels available to the cell. These models are consistent with similar models describing the actions of aldosterone on  $\text{Na}^+$  channels (Schafer and Hawk, 1992).

compelling evidence for or against the insertion model.

## 4.4 Conclusions

In conclusion, our data showed that aldosterone increases  $\text{Na}^+$  transport across cultured A6 cells mainly by changes in channel densities and not by changes to the channel kinetics or open probability. We can offer no evidence towards a mechanism whereby these changes are brought about except that our data indicated a large increase in the pool of open and closed channels.

In light of our findings, we intend to expand our investigations into possible mechanisms whereby the changes in channel densities may be produced. The most direct evidence should come from immunocytochemical studies and our laboratory is geared to perform these experiments. However, there are still many concerns regarding the specificity of the antibodies used in the current studies. Most antibodies produced so far are directed against the  $\text{Na}^+$  channel amiloride-binding site of which there may be more than one per channel, and this could be the reason why the cytochemical and biophysics data are presently not compatible. The future interest of our laboratory is directed towards analysis of vesicle trafficking in the cells. We have already examined certain aspects regarding the involvement of the cytoskeleton in the hormonal control of channel trafficking (Els and Chou, 1993; Chou and Els, 1996) and these studies should be extended to investigate their role in the aldosterone response. Ideally, vesicle trafficking should be approached by a multidisciplinary approach using a variety of techniques.

## Chapter 8

## References

---

---

- Abramcheck, F. J., Van Driessche, W. and Helman, S. I. (1985) Autoregulation of apical membrane Na<sup>+</sup> permeability of tight epithelia. Noise analysis with amiloride and CGS 4270. *Journal of General Physiology* **85**: 555-582.
- Asher, C. and Garty, H. (1988) Aldosterone increases the apical Na<sup>+</sup> permeability of toad bladder by two different mechanisms. *Proceedings of the National Academy of Sciences USA* **85**: 7413-7417.
- Asher, C., Eren, R., Kahn, L., Yeger, O. and Garty, H. (1992) Expression of the amiloride-blockable Na<sup>+</sup> channel by RNA from control *versus* aldosterone-stimulated tissue. *The Journal of Biological Chemistry* **267** (23): 16061-16065.
- Baldwin, K. S. and Els, W. J. (1995) Na<sup>+</sup> channel regulation by aldosterone in cultured renal epithelia. *Proceedings of The 23rd Annual Congress of the Physiology Society of Southern Africa* (Abstract).
- Barry, G. (1987) Structure, Biochemistry, and Assembly of Epithelial Tight Junctions. *American Journal of Physiology* **253** (Cell Physiology 22): C749 - C758.
- Bastl, C. P. and Hayslett, J. (1992) The cellular action of aldosterone in target epithelia. *Kidney International* **42**: 250-264.
- Baxendale, L. M., Duncan, R. L. and Helman, S. I. (1987) Aldosterone increases apical membrane Na<sup>+</sup> channel density in A6 epithelia (Abstract). *Federation Proceedings* **46**:495.
- Benos, D. J., Saccomani, G. and Sariban-Sohraby, S. (1987) The epithelial sodium channel. Subunit number and location of the amiloride binding site. *The Journal of Biological Chemistry* **262**:10613-10618.
- Bindels, R. J., Schafer, J. A. and Reif, M. C. (1988) Stimulation of sodium transport by aldosterone and arginine vasotocin in A6 cells. *Biochimica et Biophysica Acta* **972**: 320-330.

- Blazer-Yost, B., Geheb, M., Preston, A., Handler, J. and Cox, M. (1982) Aldosterone-induced Proteins in Renal Epithelia. *Biochimica et Biophysica Acta* **719**: 158-161.
- Bradbury, N. A. and Bridges, R.J. (1994) Role of membrane trafficking in plasma membrane solute transport. *American Journal of Physiology* **261**: C882-C888.
- Brown, K. M. and Dennis, J. E. (1972) Derivative free analogues of the Levenburg-Marquardt and Gauss algorithms for non-linear least squares approximation. *Numerische Mathematik* **18**: 289-297.
- Chou, K-Y. and Els, W. J. (1996) Effects of disassembly of actin microfilaments on avp-induced regulation of frog skin epithelium sodium channels. *Journal of Experimental Biology* (Submitted).
- Chu, L.L.H. and Eldelman, I.S. (1975) Cordycepin and alpha-amanitin: Inhibitors of transcription as probes of aldosterone action. *Journal of Membrane Biology* **10**: 291-310.
- Civan, M.M. and Hoffman, R.E. (1971) Effect of aldosterone on electrical resistance of toad bladder. *American Journal of Physiology* **220**: 324-328.
- Cooley, J. W., Lewis, P. A. W. and Welch, P. D. (1967) The fast Fourier transform and its applications. *IBM Research RC 1743*: 15-33.
- Crabbé, J. (1967) Suppression by amphotericin B of the effect exerted by aldosterone on active sodium transport. *Archives internationales de Physiologie et de Biochimie* **75**: 342-345.
- Crabbé, J. (1961) Stimulation of active sodium transport by the isolated toad bladder with aldosterone *in vitro*. *Journal of Clinical Investigation* **40**: 2103-2110.
- Cuthbert, A. W. and Shum, W.K. (1975). Effects of vasopressin and aldosterone on amiloride binding in toad bladder epithelial cells. *Proceedings of the Royal Society, London*. **189**: 543-575.
- Cuthbert, A. W. and Shum, W.K. (1978). Induction of transporting sites in a sodium transporting epithelium. *Journal of Physiology, London* **260**: 223-235.
- De Wolf, I. and Van Driessche, W. (1986) Voltage-dependent Ba<sup>++</sup> block of K<sup>+</sup> channels in apical membrane of frog skin. *American Journal of Physiology* **251**: C696-C706.

- Desmedt, L., Simaels, J. and Van Driessche, W. (1993)  $\text{Ca}^{++}$ -blockable, poorly selective cation in the apical membrane of amphibian epithelia.  $\text{UO}_2^{++}$  reveals two channel types. *Journal of General Physiology* **101**: 85-102.
- Diamond, J. M. (1978) Solute-linked water transport in epithelia. In: *Membrane Transport Processes*. Edited by J.F. Hoffmann, Raven Press, New York. 257-276.
- Eaton, D.C., Becchetti, A., Ma, H., Ling, B.N. (1995) Renal sodium channels: Regulation and single channel properties. *Kidney International* **48**: 941-949.
- Edelman, I.S., Bogoroch, R. and Porter, G.A. (1963) On the mechanism of action of aldosterone on sodium transport: the role of protein synthesis. *Proceedings of the National Academy of Sciences USA* **50**: 1169-1177.
- Edelman, I. S. (1981) Receptors and effectors in hormone action on the kidney. *American Journal of Physiology* **241**: F333-F339.
- Els, W. J. and Helman, S. I. (1991) Activation of epithelial Na channels by hormonal and autoregulatory mechanisms of action. *Journal of General Physiology* **98**: 1197-1220.
- Els, W. J. and Chou, K.-Y. (1993) Sodium-dependent regulation of epithelial sodium channel densities in frog skin; a role for the cytoskeleton. *Journal of Physiology* **462**: 447-464.
- Els, W. J. and Helman, S.I. (1996) Dual Role of Prostaglandins ( $\text{PGE}_2$ ) in regulation of channel densities and open probability of epithelial  $\text{Na}^+$  channels in frog skin (*R. pipens*). *Journal of Membrane Biology* (Submitted).
- Els, W. J. and Helman, S. I. (1989) Regulation of sodium channel densities by vasopressin signalling. *Cellular Signalling* **1**(6): 533-539.
- Fidelman, M.L. and Watlington, C.O. (1987) Effect of aldosterone and insulin on mannitol,  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes in cultured epithelia of renal origin (A6): evidence for increased permeability in the paracellular pathway. *Biochimica et Biophysica Acta* **931**: 205-214.
- Fischer, H. and Clauss, W. (1990) Regulation of  $\text{Na}^+$  channels in frog lung epithelium: a target tissue for aldosterone action. *Pflügers Archiv European Journal of Physiology* **416**: 62-67.
- Frindt, G., Sackin, H. and Palmer, L. G. (1990) Whole-cell currents in rat cortical collecting tubule: Low-Na diet increases amiloride-sensitive conductance. *American Journal of Physiology* **258** (27): F562-F567.

- Frizzel, R.A. and Schultz, S.G. (1978) Effect of aldosterone on ion transport by rabbit colon in vitro. *Journal of Membrane Biology* **39**: 1-26.
- Ganong, W. F. and Murlow, P. J. (1958) Rate of change in sodium and potassium excretion after injection of aldosterone into the aorta and renal artery of the dog. *American Journal of Physiology* **195**: 337-342.
- Garty, H. and Benos, D. J. (1988) Characteristics and regulatory mechanisms of the amiloride-blockable Na<sup>+</sup> channels. *Physiological Reviews* **68**: 309-373.
- Garty, H. and Edelman, I. S. (1983) Amiloride-sensitive trypsinization of apical sodium channels. Analysis of hormonal regulation of sodium transport in toad bladder. *Journal of General Physiology* **81**: 785-803.
- Garty, H. (1986) Mechanisms of aldosterone action in tight epithelia. *Journal of Membrane Biology* **90**: 193-205.
- Garty, H. (1994) Molecular properties of epithelial, amiloride-blockable Na<sup>+</sup> channels. *FASEB Journal* **8**: 0522-0528.
- Geheb, M., Huber, G., Hercker, E. and Cox, M. (1981) Aldosterone-induced Proteins in Toad Urinary Bladders. *The Journal of Biological Chemistry* **256** (22): 11716-11723.
- Granitzer, M., Mountain, I. and Van Driessche, W. (1995) Effect of dexamethasone on sodium channel block and densities in A6 cells. *Pflügers Archiv European Journal of Physiology* **430**: 493-500.
- Hamilton, K. L. and Eaton, D. C. (1985) Single-channel recordings from amiloride-sensitive epithelial sodium channel. *American Journal of Physiology* **249**: C200-C207.
- Handler, J. S. (1988) Antidiuretic hormone moves membranes. *American Journal of Physiology* **255**: F375-F382.
- Handler, J.S., Perkins, F.M. and Johnson, J.P. (1980) Studies of renal cell function using cell culture techniques. *American Journal of Physiology* **238**: F1-F9.
- Helman, S.I. (1973) Microelectrode studies of isolated cortical collecting tubules (Abstract). *Proceedings: Annual Meeting of the American Society of Nephrology, 6th, Washington, DC*, p49.
- Helman, S. I. (1979) Electrochemical potentials in frog skin: inferences for electrical and mechanistic models. *Federation Proceedings* **38**(13): 2743-2750.

- Helman, S. I. and Baxendale, L. M. (1990) Blocker-related changes of channel density: Analysis of a three-state model for apical Na channels of frog skin. *Journal of General Physiology* **95**: 647-678.
- Helman, S. I. and Fisher, R. S. (1977) Microelectrode studies of the active Na transport pathway of frog skin, *Journal of General Physiology* **69**: 571-604.
- Helman, S. I., Koeppen, B. M., Beyenbach, K. W. and Baxendale, L. M. (1985) Patch clamp studies of apical membranes of renal cortical collecting ducts. *Pflügers Archiv European Journal of Physiology* **405** (Suppl. 1): S71-76.
- Helman, S. I. and Kizer, N. L. (1990) Apical sodium ion channels of tight epithelia as viewed from the perspective of noise analysis. In: *Current Topics in Membrane and Transport*, edited by Bronner, F. Academic Press, Inc. **37**: 117-155.
- Helman, S. I. and Miller, D. A. (1971) In vitro techniques for avoiding edge damage in studies of frog skin. *Science* **173**: 146-148.
- Helman, S. I. and Van Driessche, W. (1990) Channels and noise in epithelial tissues. In: *Current Topics in Membranes and Transport*, edited by Bronner, F. Academic Press, Inc. Volume **37**.
- Hong, C.D., and Essig, A. (1976) Effects of 2-deoxy-D-glucose, amiloride, vasopressin and ouabain on active conductance and  $E_{Na}$  in the toad bladder. *Journal of Membrane Biology* **28**: 121-142.
- Horisberger, J.-D. (1992) Early effects of aldosterone on apical and basolateral membrane conductances of TBM cells. *American Journal of Physiology* **263** (Cell Physiology 32): C384- C388.
- Ismailov, I. I., McDuffie, J. H., Sariban-Sohraby, S., Johnson, J. P. and Benos, D. J. (1994) Carboxyl methylation activates purified renal amiloride-sensitive  $Na^+$  channels in planar lipid bilayers. *The Journal of Biological Chemistry* **269** (35): 22193-22197.
- Kemendy, A. E. and Eaton, D. C. (1990) Aldosterone-induced  $Na^+$  transport in A6 cells is blocked by 3-deazaadenosine, a methylation blocker. (abstract) *FASEB Journal* **4**: A445
- Kemendy, A. E., Kleyman, T. R. and Eaton, D. C. (1992) Aldosterone alters the open probability of amiloride-blockable sodium channels in A6 epithelia. *American Journal of Physiology* **263** (Cell Physiology 32): C825-C837.

- Kizer, N.L., Lewis, B. and Stanton, B.A. (1995) Electrogenic sodium absorption and chloride secretion by an inner medullary collecting duct cell line (mIMCD-K2). *American Journal of Physiology* **268**: F347-355.
- Kleyman, T. R., Cragoe, E. J., Jr. and Kraehenbühl, J.-P. (1989) The cellular pool of Na<sup>+</sup> channels in the amphibian cell line A6 is not altered by mineralocorticoids. Analysis using a new photoactive amiloride analog in combination with anti-amiloride antibodies. *The Journal of Biological Chemistry* **264**: 11995-12000.
- Kleyman, T. R., Coupaye-Gerard, B. and Ernst, S. A. (1991) Aldosterone does not alter the cell-surface expression of epithelial Na<sup>+</sup> channels in the amphibian cell line A6. *The Journal of Biological Chemistry* **267**: 9622-9628.
- Koefoed-Johnsen, V. and Ussing, H. H. (1958) The nature of the frog skin potential. *Acta Physiologica Scandinavica* **42**: 298-308.
- Koeppen, B. M., Biagi, B. A. and Giebisch, G. H. (1983) Intracellular microelectrode characterization of the rabbit cortical collecting duct. *American Journal of Physiology* **244** (Renal fluid electrolyte physiology 13): F35-F47.
- Lahav, M., Dietz, T. and Edelman, I.S. (1973) The action of aldosterone on sodium transport: Further studies with inhibitors of RNA and protein synthesis. *Endocrinology* **92**: 1685-1699
- Leaf, A. and MacKnight, A.D.C. (1972) The site of the aldosterone induced stimulation of sodium transport. *Journal of Steroid Biochemistry* **3**: 237-245.
- Lewis, S. A. and Wills, N. K. (1983) Apical membrane permeability and kinetic properties of the sodium pump in rabbit urinary bladder. *Journal of Physiology* **341**: 169-184.
- Lewis, S.A., and Donaldson P.J. (1990) Patch clamp of cation channels. In: *Current Topics in Membrane and Transport*, edited by Bronner, F. Academic Press, Inc. **37**: 215-246.
- Lewis, S.A. and de Moura, J.L.C. (1984). Apical membrane area of rabbit urinary bladder increases by fusion of intracellular vesicles: An electrophysiological study. *The Journal of Experimental Biology* **82**: 123-136.
- Lindemann, B. and Van Driessche, W. (1977) Sodium-specific membrane channels of frog skin are pores: current fluctuations reveal high turnover. *Science* **195**: 292-294.
- Lipton, P. and Edelman, I.S. (1971) Effects of aldosterone and vasopressin on electrolytes of toad bladder epithelial cells. *American Journal of Physiology* **221**: 733-741.

- Liu, X., Els, W. J. and Helman, S. I. (1995) Aldosterone increases the apical membrane capacitance of A6 epithelia (*Abstract*) *FASEB* 9: A64 375.
- Marunaka, Y. and Eaton, D. C. (1991) Effects of vasopressin and cAMP on single amiloride-blockable Na channels. *American Journal of Physiology* 260: C1071-1084.
- Minuth, W. W., Steckelings, U. and Gross, P. (1987) Complex physiological and biochemical action of aldosterone in toad urinary bladder and mammalian renal collecting duct cells. *Renal Physiology* 10: 297-310.
- Nagel, W. and Katz, U. (1991) The effect of aldosterone on sodium transport and membrane conductances in toad skin (*Bufo viridis*). *Pflügers Archiv European Journal of Physiology* 418: 319-324.
- Nagel, W. and Crabbé, J. (1980) Mechanism of action of aldosterone on active sodium transport across toad skin. *Pflügers Archiv European Journal of Physiology* 385: 181-187.
- Pácha, J., Frindt, G., Antonian, L., Silver, R.B. and Palmer, L. G. (1993) Regulation of Na channels of the rat collecting tubule by aldosterone. *Journal of General Physiology* 102: 25-42.
- Palmer, L. G. and Frindt, G. (1980) Epithelial sodium channels: characterization by using the patch-clamp technique. *Federation Proceedings* 45: 2708-2712.
- Palmer, L.G. and Sackin, H. (1988) Regulation of renal ion channels. *FASEB Journal* 2: 3061-3065.
- Palmer, L. G. and Frindt, G. (1986b) Amiloride-sensitive Na channels from the apical membrane of the rat cortical collecting tubule. *Proceedings of the National Academy of Sciences USA* 83: 2767-2770.
- Palmer, L. G., Li, H-Y., Lindemann, B. and Edelman, S. I. (1982) Aldosterone control of the density of sodium channels in the toad urinary bladder. *Journal of Membrane Biology* 57: 59-71.
- Palmer, L. G. and Frindt, G. (1986a) Epithelial sodium channel: characterisation by using the patch-clamp technique. *Federation Proceedings* 45: 2708-2712.
- Rafferty, R.A. (1969) Mass culture of amphibian cells: Methods and observations concerning stability of cell type. In: *Biology of of Amphibian Tumors*, edited by Mizell, M. Springer-Verlag, New York: 52-81.

- Rossier, B. C., Paccolat, M.-P., Verrey, F., Kraehenbuhl, J.-P. and Geering, K. (1985) Mechanism of action of aldosterone: A pleiotropic response. In: *Hormones and Cell Regulation*, edited by Dumont, J.E., Hamprecht, B. and Nunez, J., INSERM, Paris 9: 209-225.
- Rossier, B. C., Wilce, P.A. and Edelman, I.S. (1974) Kinetics of RNA labeling in toad bladder epithelium: Effects of aldosterone and related steroids. *Proceedings of the National Academy of Sciences USA* 71: 3101-3105.
- Rossier, B.C., Verrey, F. and Kraehenbuhl, J-P. (1988) Transepithelial sodium transport and its control by aldosterone: a molecular approach. *Current Topics in Membranes and Transport* 34: 167-183.
- Ruknudin, A., Song, M. J. and Sachs, F. (1991) The ultrastructure of patch-clamped membranes: A study using high voltage electron microscopy. *The Journal of Cell Biology* 112: 125-134.
- Saito, T. and Essig, A. (1973) Effect of aldosterone on active and passive conductance and  $E_{Na}$  in the toad bladder. *Journal of Membrane Biology* 13: 1-18.
- Sansom, S. C. and O'Neil, R.G. (1985) Mineralocorticoid regulation of apical membrane  $Na^+$  and  $K^+$  transport of the cortical collecting duct. *American Journal of Physiology* 248: F858-F868.
- Sariban-Sohraby, S., Mies, F., Abramow, M. and Fisher, R.S. (1995) Aldosterone stimulation of GTP hydrolysis in membranes from renal epithelia. *American Journal of Physiology* 268 (Cell Physiology, 37): C557-C562.
- Sariban-Sohraby, S., Burg, M.B., and Turner, R.J. (1984a) Aldosterone-stimulated sodium uptake by apical membrane vesicles from A6 cells. *The Journal of Biological Chemistry* 259 (18): 11221-11225.
- Sariban-Sohraby, S., Burg, M., Wiesmann, W.P., Chaing, P.K. and Johnson, J.P. (1984b) Methylation increases  $Na^+$ -transport into A6 apical membrane vesicles: possible mode of aldosterone action. *Science* 225: 745.
- Sariban-Sohraby, S., Fisher, R.S. and Abramow, M. (1993) Aldosterone-induced and GTP-stimulated methylation of a 90 kD polypeptide in the apical membrane of A6 epithelial cells. *The Journal of Biological Chemistry* 268: 26613--26617.
- Sariban-Sohraby, S. and Fisher, R.S. (1995) Guanine nucleotide-dependent carboxy-methylation: A pathway for aldosterone modulation of apical  $Na^+$  permeability in epithelia. *Kidney International* 48: 965-969.

- Schafer, J. A. and Hawk, C. T. (1992) Regulation of Na<sup>+</sup> channels in the cortical collecting duct by AVP and mineralocorticoids. *Kidney International* **41**: 255-268.
- Schwartz, G. J. and Burg, M. B. (1978) Mineralocorticoid effects on cation transport by cortical collecting tubules in vitro. *American Journal of Physiology* **235**: F576-F585.
- Sharp, G.W.G. and Leaf, A. (1966) Mechanism of action of aldosterone. *Physiological Reviews* **46** (4): 593-633.
- Simpson, S.A., Tait, J.F. and Bush, I.E. (1952) Secretion of a salt-retaining hormone by the mamalian adrenal gland. *Lancet* **263**: 226-227.
- Spooner, P. M. and Edelman, I. S. (1975) Further studies on the effect of aldosterone on electrical resistance of toad bladder. *Biochimica et Biophysica Acta* **406**: 304-314.
- Szerlip, H. M., Weisberg, L., Clayman, M., Neilson, E., Wade, J. B. and Cox, M. (1989) Aldosterone-induced proteins: purification and localization of GP65, 70. *American Journal of Physiology* **256**: C865-C872.
- Tousson, A., Alley, C. D., Sorscher, E. J., Brinkley, B. R. and Benos, D. J. (1989) Immunochemical localization of amiloride-sensitive Na<sup>+</sup> channels in sodium transporting epithelia. *Journal of Cell Science* **93**: 349-62.
- Tsakiridis, T., Vranic, M. and Klip, A. (1994) Disassembly of the actin network inhibits insulin-dependent stimulation of glucose transport and prevents recruitment of glucose transporters to the plasma membrane. *The Journal of Biological Chemistry* **269**: 29934-29942.
- Turnheim, K. (1994) Epithelial sodium transport: basic autoregulatory mechanisms. *Physiological Research* **43**: 211-218.
- Ussing, H. H. and Zerahn, K. (1951) Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiologica Scandinavica* **23**: 110-127.
- Van Driessche, W. and Gullentops, K. (1982) Conductance fluctuation analysis in epithelia. In: *Techniques in the life sciences. Techniques in Cellular Physiology* edited by Baker, P. F., Elsevier/North-Holland Scientific Publishers, Amsterdam **2**: 1-13.
- Van Driessche, W. and Lindemann, B. (1978) Low-noise amplification of voltage and current fluctuations arising in epithelia. *Reviews of Scientific Instrument* **49**: 52-57.

- Verrey, F. (1994) Antidiuretic hormone action in A6 cells: Effect on apical Cl and Na conductances and synergism with aldosterone for NaCl reabsorption. *Journal of Membrane Biology* **138**: 65-76.
- Verrey, F. (1995) Transcriptional control of sodium transport in tight epithelia by adrenal steroids. *Journal of Membrane Biology* **144**: 93-110.
- White, P.C. (1994) Disorders of aldosterone biosynthesis and action. *The New England Journal of Medicine* **331**(4): 250-258.
- Wiesmann, W. P., Johnson, J. P., Miura, G. A. and Chiang, P. K. (1985) Aldosterone-stimulated transmethylations are linked to sodium transport. *American Journal of Physiology* **248**: F43-F47.
- Wiesmann, W.P., Chiang, P.K. and Johnson, J.P. (1983) Aldosterone stimulates phospholipid methylations in cultured toad urinary bladder epithelial cells. *Clinical research* **31**: 445A.
- Wills, N. K. and Zweifach, A. (1987) Recent advances in the characterisation of epithelial ionic channels. *Biochimica et Biophysica Acta* **906**: 1-32.
- Wills, N.K., Purcell, R.K., Clausen, C. and Millinoff, L.P. (1993) Effects of aldosterone on the impedance properties of cultured renal amphibian epithelia. *Journal of Membrane Biology* **133**: 17-27.
- Wills, N. K., Purcell, R. K., Clausen, C. and Millinoff, L. P. (1993) Effects of aldosterone on the impedance properties of cultured renal amphibian epithelia. *Journal of Membrane Biology* **133**: 17-27.
- Wills, N.K., Millinoff, L.P. and Crowe, W.E. (1991) Na<sup>+</sup> channel activity in cultured renal (A6) epithelium: Regulation by solution osmolarity. *Journal of Membrane Biology* **121**: 79-90.
- Wills, N. K. and Millinoff, L. P. (1990) Amiloride-sensitive Na<sup>+</sup> transport across cultured renal (A6) epithelium: evidence for large currents and high Na:K selectivity. *Pflügers Archiv European Journal of Physiology* **416**: 481-492.

## Appendix

### ***Cell Culture Freezing Down Procedure***

Cells maintained on flasks were lifted as described in *Materials and Methods* and the contents of the flasks were transferred to 10 ml centrifuge tubes. Cells were spun down at 1000 g for 2 minutes and re-suspended in Origen DMSO freezing medium (10 % DMSO and 20 % FCS in DMEM). 1 ml aliquots were pipetted into cryovials which were then placed in a freezer at -5 °C overnight, after which they were transferred into liquid nitrogen.

### ***Electrodes***

The current and voltage electrodes were made with silver (Ag) wires, which were scraped to remove oxides and then electrocoated with chloride ( $\text{Cl}^-$ ) ions. This was achieved by placing the Ag wire into a beaker containing a 1:50 hydrochloric acid (HCl) solution and attaching the wire to the anode. The cathode was placed in the same beaker a little distance away from the Ag wire and a voltage of about 2V was applied across the termini for approximately 10 minutes until the wire was coated evenly with  $\text{Cl}^-$  ions - a black coating. 5M NaCl/agar (2%) (May & Baker Ltd., U.K.) bridges were used to prepare four Ag/AgCl electrodes. Two electrodes served as current electrodes, while the other two were voltage electrodes. The inter-electrode potentials were measured with a multimeter and kept as low as possible (<1 mV) in order to minimise false measurements of the  $I_{sc}$ . This was again checked and recorded at the end of the experiment. KCl is often used in the preparation of the agar bridges because the motilities of the two ion species are very similar, whereas the motilities of Na and Cl are not so close. However 5 M NaCl was preferred to KCl as any leaking of KCl would influence the system more seriously than would NaCl (Van Driessche and Erji, 1983).

